The MAP kinase phosphatase, MKP-1, regulates BDNF-induced axon branching

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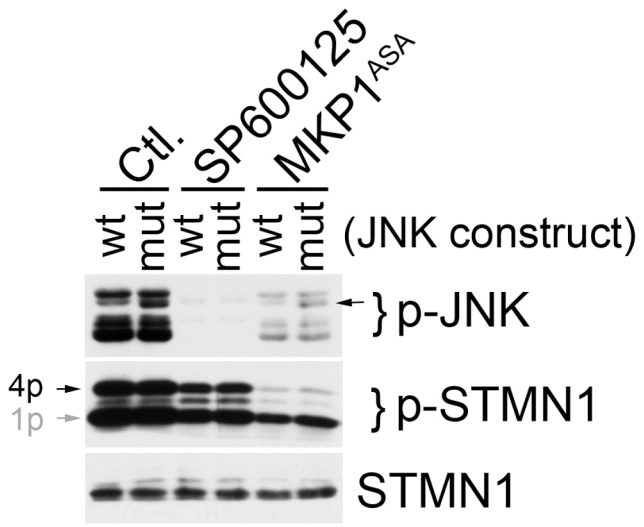
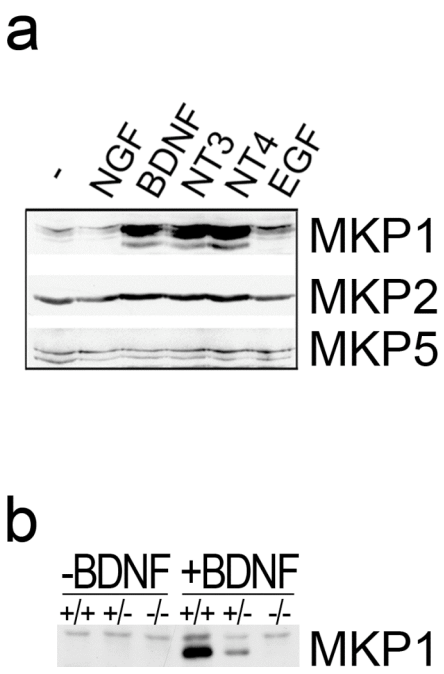
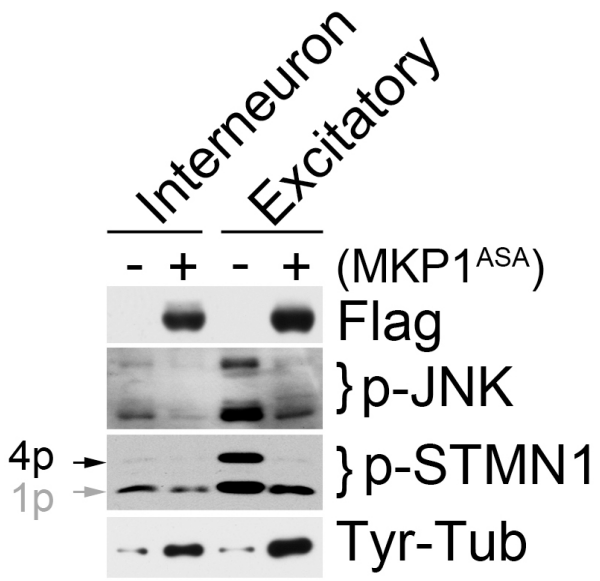


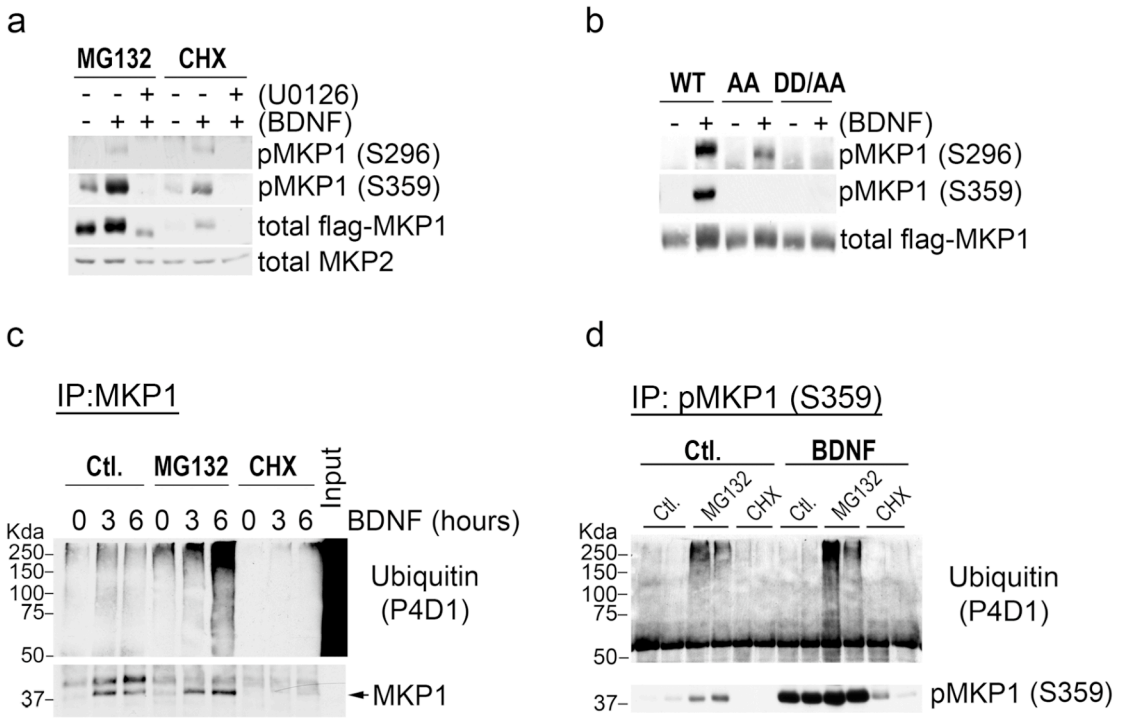
Supplementary figure 1. Characterization of the JNK$^{R69S/D326N}$ double mutant in neurons. The mutations R65S and D319N within the MAPK Erk1/2 sequence have been previously reported to increase constitutive activity[23] and decrease sensitivity to MKP-3[22], respectively. These mutations have been transposed to JNK (R69S and D326N) based on sequence homology. Cortical neurons were electroporated with the JNK wild type or mutant JNK$^{R69S/D326N}$ constructs together with MKP-1^{ASA} or an empty plasmid. Cells were grown for 5 d before treatment with the JNK inhibitor (SP600125, 10 μM for 2 h). Lysates were tested for phosphorylation of JNK and its substrate, STMN1, by Western blot. The arrow indicates the band corresponding to the transfected JNK$^{R69S/D326N}$. Despite a better blockade of the pJNK signal with the JNK inhibitor compared to MKP-1^{ASA}, the reduction of pSTMN1 (S25-P) by the ectopic expression of MKP-1^{ASA} for 5 d exceeded that of SP600125 for 2 h. The JNK$^{R69S/D326N}$ mutant slightly increased p-STMN1 phosphorylation despite co-expression of MKP-1^{ASA}, indicating that this mutant is not completely insensitive to MKP-1. 1p and 4p indicate the mono- and multi-phosphorylated forms of STMN1, respectively. Western blots are representative images from 3 independent experiments.



Supplementary figure 2. Induction of MKP-1 by several trophic factors. (**a**) Cortical neurons grown in culture (DIV5) were stimulated with the indicated trophic factors (50 ng/ml) for 3 h. Lysates were tested for MKP-1 expression levels by Western blot. Related MKP family members, MKP-2 and MKP-5 (rabbit polyclonal antibodies from Abgent) were also tested for expression changes. (**b**) Validation of MKP-1 antibody in primary E18 cortical cultures from MKP-1 knockout mice. Western blots with the MKP-1 antibody (Santa Cruz, M-18) showed two bands (≈ 39-40 Kda and ≈ 42 Kda) in mouse and rat neuronal culture lysates. The top band (42 Kda) is not specific and not inducible by BDNF treatment (50 ng/ml for 3 h).

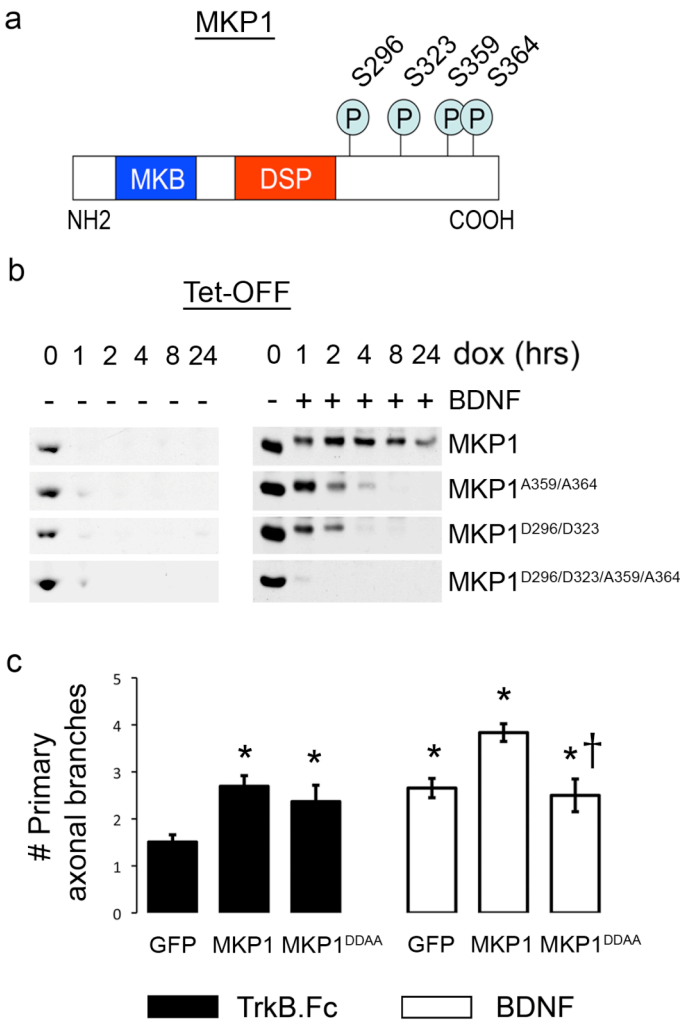


Supplementary figure 3. Similar mode of action of MKP-1^{ASA} in inhibitory and excitatory neurons. Ectopic expression of flag-MKP-1^{ASA} between DIV 1–5 using lentivirus in sorted GFP-positive interneurons and GFP-negative excitatory neurons decreased pJNK and pSTMN1 (S25-P) but increased tubulin tyrosination similarly in both cell types. 1p and 4p indicate the mono- and multi-phosphorylated forms of STMN1, respectively. Observations were reproduced at least 3 times in 2 independent experiments.



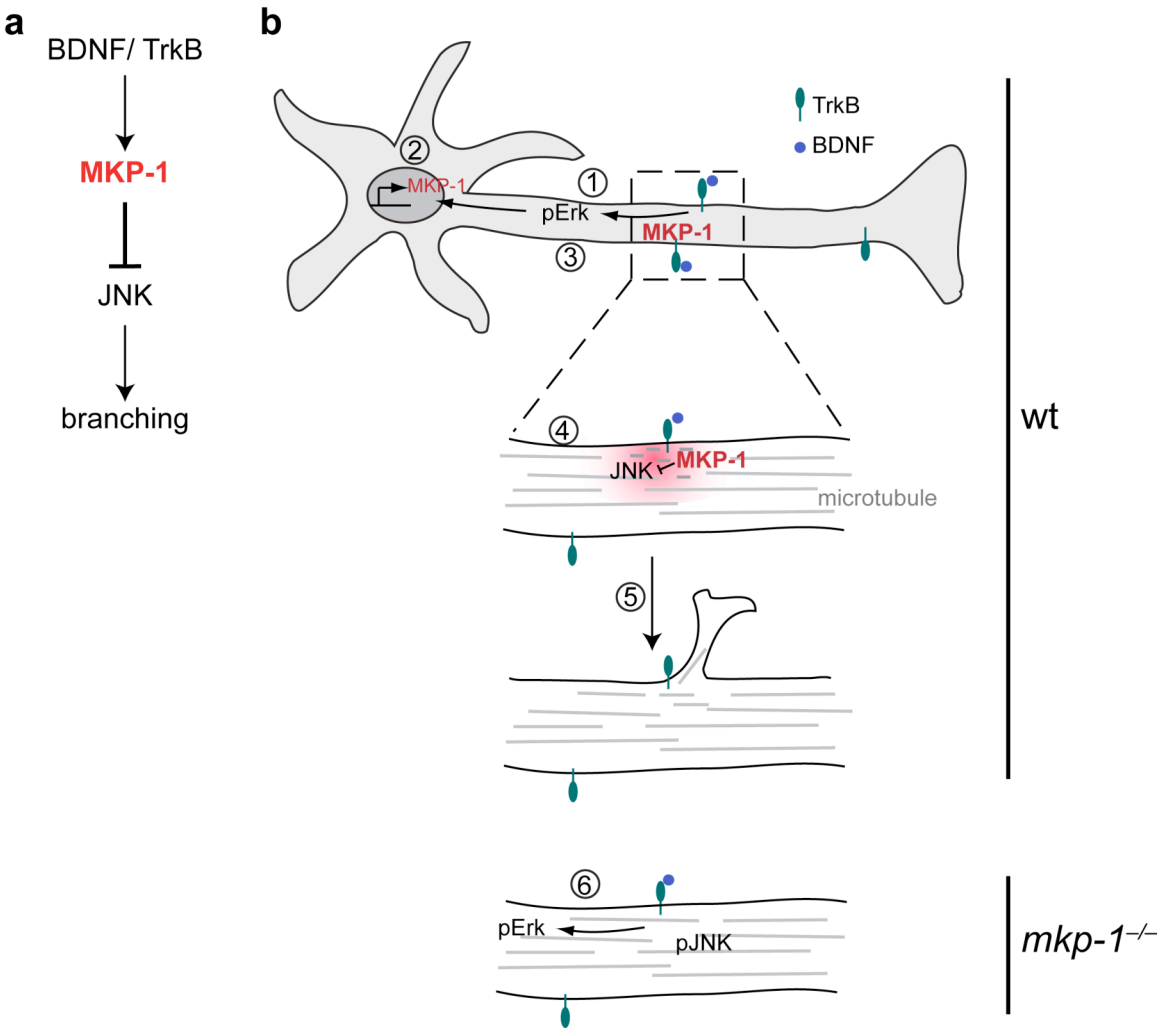
Supplementary figure 4. Neuronal MKP-1 turnover is controlled by a BDNF/Erk signaling pathway. (**a**) Turnover of MKP-1 protein in neurons is impaired by BDNF signaling via the Erk1/2 pathway. Cortical neurons electroporated with the flag-tagged MKP-1 were treated with MG132 (10 μM) or cycloheximide (CHX, 20 μg/ml) for 2 h to prevent or accelerate, respectively, the clearance of the transfected MKP-1 protein monitored in cell lysates with anti-flag antibodies. Stimulation with BDNF (50 ng/ml) for 2 h sustained flag-MKP-1 protein level, which was blocked by co-treatment with the MEK1/2 inhibitor, U0126 (10 μM). Phosphorylation of MKP-1 (at two distinct sites S296 and S359) correlated with total MKP-1 protein level, which was increased by BDNF stimulation and decreased by co-treatment with U0126. (**b**) Validation of MKP-1 phospho-specific antibodies. 293/TrkB cells transfected with flag-tagged MKP-1 constructs were stimulated with BDNF (50 ng/ml) for 30 minutes. Note that mutations of serine 359 into alanine (phospho-deficient MKP-1$^{A359/A364}$ mutant termed AA) and serine 296 into glutamate (phospho-mimicking mutation combined with the AA mutations to produce a quadruple mutant termed DD/AA: MKP-1$^{D296/D323/A359/A364}$) abolished the signals detected by the phospho-specific MKP-1 antibodies. (**c**) Cortical neurons treated

with MG132 (10 μM) or cycloheximide (CHX, 20 μg/ml) for 6 h were stimulated with BDNF (50 ng/ml) for the indicated time. Lysates were immunoprecipitated with MKP-1 antibodies and tested for ubiquitination in Western blot. Note that MG132 treatment alone for 6 h did not significantly raise MKP-1 expression level when compared to untreated control, suggesting that BDNF signaling both induces and stabilizes MKP-1 expression. (**d**) No correlation exists between phosphorylation of residue serine 359 and ubiquitination of MKP-1. Endogenous MKP-1 from the 293/TrkB stable cell line was immunoprecipitated using phospho-specific MKP-1 antibodies (S359-P). Treatment with BDNF (50 ng/ml) for 3 h increased MKP-1 phosphorylation at residue S359. When phosphorylated on residue S359, immunoprecipitated MKP-1 was detected either as ubiquitinated in the presence of MG132 and not ubiquitinated in the absence of MG132. Therefore, phosphorylation of MKP-1 at the S359 site alone cannot be a competition signal for ubiquitination.



Supplementary figure 5. BDNF-induced stabilization of MKP-1 expression is controlled by a sophisticated phosphorylation signature within the MKP-1 C-terminal domain. (a) Schematic representation of MKP-1 protein domain organization. MKB, MAPK-binding domain; DSP, dual specificity phosphatase domain. The C-terminal domain of MKP-1 is phosphorylated on several sites. Phosphorylation on both S359 and S364 delay MKP-1 degradation whereas phosphorylation on both S296 and S323 enhance MKP-1 degradation, as previously described[32,33]. We found that BDNF signaling triggered MKP-1 phosphorylation on both S296 and S359 via a Erk-dependent mechanism, as well as a sustained MKP-1 protein expression. (b) To address the role of BDNF-dependent phosphorylation upon MKP-1 protein stabilization, we generated a series of mutations that made MKP-1 insensitive to BDNF signaling. We took advantage

of the inducible tet^{OFF} system to uncouple expression of wildtype and mutant MKP-1 from BDNF signaling. In this experimental set up (described in Fig. 4b), addition of doxycyclin (DOX) turned off MKP-1 expression. In transfected 293/TrkB cells, MKP-1 turnover was prolonged upon BDNF treatment with the wild-type construct but not when both S359 and S364 were mutated into alanine and both S296 and S323 were changed into glutamate. The resulting quadruple mutant MKP-$1^{D296/D323/A359/A364}$ was insensitive to BDNF signaling whereas individual mutations of both the S359 and S364 into alanine or both the S296 and S323 into glutamate only decreased the stabilization effect of BDNF. Consequently, MKP-1 protein turnover is controlled in a BDNF-dependent fashion by a sophisticated phosphorylation signature. (c) Characterization of the effect of the MKP-$1^{DD/AA}$ quadruple mutant on axon branching. The experimental procedure described in Fig. 5a was applied to test the MKP-$1^{DD/AA}$ mutant. BDNF treatment synergized with MKP-1 to further increase axon primary branching, an effect lost with the MKP-$1^{DD/AA}$ mutant (†, $p=0.003$, t-test). Therefore, if the ectopic expression of MKP-1 or MKP-$1^{DD/AA}$ mutant alone is sufficient to elicit axon branching (all groups compared to GFP, *, $p<0.05$, t-test), the effect of BDNF depended on the prolonged stabilization of MKP-1. The number of primary axonal branches was expressed as mean ± s.e.m. The number of cells analyzed for the TrkB.Fc group was as following: GFP (n= 71), MKP-1 (n= 78), MKP-1^{DDAA} (n= 30), and for the BDNF group: GFP (n= 87), MKP-1 (n= 109), MKP-1^{DDAA} (n= 22). All experiments were conducted at least 3 times.



Supplementary figure 6. Controlled MKP-1 activity facilitates axonal branch formation. (**a**) Flowchart of the key signaling pathway involved in BDNF-induced axon branching. (**b**) Model: (**1**) Retrograde BDNF-TrkB signaling via pErk induces MKP-1 expression. (**2**) Transient induction of MKP-1 from low basal levels, coupled with a high turnover rate, is a mechanism to constrain its activity. (**3**) MKP-1 can be phosphorylated through continued BDNF signaling, which in turn increases its stability. (**4**) Prolonged MKP-1 activity at the sites of BDNF signaling leads to deactivation of JNK and destabilization of microtubules. (**5**) Enhanced microtubule instability facilitates cytoskeletal rearrangements and is therefore a permissive cue for BDNF-induced axon branching. (**6**) In *mkp-1*$^{-/-}$ neurons, BDNF signaling does not impact on microtubule stability and therefore does not facilitate branch formation.