Stimulation of NeuroD activity by huntingtin and huntingtin-associated proteins HAP1 and MLK2

Edoardo Marcora, Katherine Gowan, and Jacqueline E. Lee†

Department of Molecular, Cellular, and Developmental Biology, University of Colorado, Campus Box 347, Boulder, CO 80309

Communicated by William B. Wood, University of Colorado, Boulder, CO, June 3, 2003 (received for review April 10, 2003)

NeuroD (ND) is a basic helix–loop–helix transcription factor important for neuronal development and survival. By using a yeast two-hybrid screen, we identified two proteins that interact with ND, huntingtin-associated protein 1 (HAP1) and mixed-lineage kinase 2 (MLK2), both of which are known to interact with huntingtin (Htt). Htt is a ubiquitous protein important for neuronal transcription, development, and survival, and loss of its function has been implicated in the pathogenesis of Huntington's disease, a neurodegenerative disorder. However, the mechanism by which Htt exerts its neuron-specific function at the molecular level is unknown. Here we report that Htt interacts with ND via HAP1, and that MLK2 phosphorylates and stimulates the activity of ND. Furthermore, we show that Htt and HAP1 facilitate the activation of ND by MLK2. To our knowledge, ND is the first example of a neuron-specific transcription factor involved in neuronal development and survival whose activity is modulated by Htt. We propose that Htt, together with HAP1, may function as a scaffold for the activation of ND by MLK2.

Members of the NeuroD (ND) family of basic helix–loop–helix (bHLH) transcription factors are important regulators of neuronal development and survival in vertebrates (1–6). In *Xenopus* embryos, overexpression of ND results in premature neuronal differentiation and ectopic neurogenesis (1). In mice, disruption of ND causes massive cell death in subsets of differentiating and mature neurons (2, 4, 6). ND is also involved in the development and survival of pancreatic β cells (7) and in the transcriptional activation of the insulin gene (8). Indeed, mutations in ND cause diabetes in mice and humans (7, 9). In addition, expression of ND is maintained in mature neurons and pancreatic β cells throughout adulthood, where it activates genes that contribute to the neuronal and endocrine phenotypes (5, 8, 10–12). However, how the activity of ND is controlled in different cellular contexts remains largely unknown. To identify proteins that interact with ND, we performed a yeast two-hybrid screen by using ND as bait. We found that ND interacts with two proteins that have been previously shown to interact with huntingtin (Htt), Htt-associated protein 1 (HAP1) and mixed-lineage kinase 2 (MLK2).

Htt is the protein that causes Huntington's disease (HD), a hereditary neurodegenerative disorder characterized by progressive physical and mental deterioration that ultimately leads to death (13). At the molecular level, HD is caused by the expansion of a polyglutamine tract near the N terminus of Htt (14). Despite its ubiquitous expression, mutant Htt is selectively toxic to neurons in ways not well understood (15). HAP1 was originally isolated in a yeast two-hybrid screen by using Htt as bait (16). Like Htt, HAP1 is thought to function in endocytic trafficking and vesicular transport (17, 18). MLK2 is a protein kinase that phosphorylates MKK 4/7 and consequently activates the JNK signaling pathway (19). Like HAP1, MLK2 is enriched in neurons and associated with Htt (20). However, the biological significance of these interactions is as yet unresolved.

Htt plays an important role in neuronal development (21, 22) and survival (23, 24), and it has been recently shown to regulate neuronal transcriptional events (25). However, the mechanism by which Htt exerts its neuron-specific function at the molecular level remains to be identified. One possibility is that Htt directly or indirectly interacts with a neuron-specific transcription factor involved in neuronal development and survival. Because loss of Htt function has been implicated in the etiology of HD (26–28), identification of the neuronal transcriptional pathway affected by Htt would help elucidate the molecular basis of HD (29).

Here we report that Htt interacts with ND via HAP1, and that MLK2 phosphorylates and stimulates the activity of ND. Furthermore, we show that Htt and HAP1 facilitate the activation of ND by MLK2. Our finding represents a previously undescribed case in which the activity of a neuron-specific transcription factor involved in neuronal development and survival is modulated by Htt.

Materials and Methods

Yeast Two-Hybrid Screen. The yeast two-hybrid screen was performed as in ref. 1 except for the bait plasmid that was generated by cloning a cDNA fragment corresponding to amino acids 73–232 of the mouse ND protein into the pBTM116 vector, downstream of and in-frame with the LexA DNA-binding domain. Deletion and point mutants were generated by using standard recombinant DNA methods or the QuikChange site-directed mutagenesis kit (Stratagene), as directed by the manufacturer.

Transfection and Immunoprecipitation. N2A mouse neuroblastoma cells (American Type Culture Collection nos. CCL-131) were cultured in DMEM containing 10% (vol/vol) FBS and transiently transfected by using the FuGENE 6 transfection reagent (Roche Applied Science, Indianapolis), as directed by the manufacturer, in 10-cm tissue culture dishes. One day after transfection, N2A cells were lysed with 1 ml of ice-cold 50 mM Tris (pH 7.5)/150 mM sodium chloride/1% (vol/vol) Igepal CA-630 (Sigma–Aldrich)/10% (vol/vol) glycerol/Complete protease inhibitors (EDTA-free, Roche Applied Science)/1 mM EDTA, and incubated on ice for 30 min. The lysate was then centrifuged at 16,000 $\times$ *g* for 30 min at 4°C and the supernatant immunoprecipitated with 1–5 μg of purified antibody and 50 μl of a 50% (vol/vol) protein G-agarose slurry (Roche Applied Science). After overnight incubation at 4°C on a rotator, the protein G-agarose immunocomplex was washed once with lysis buffer and three times with 50 mM Tris (pH 7.5)/300 mM sodium chloride/0.1% (vol/vol) Igepal CA-630 (Sigma–Aldrich). After the final wash, the pellet was resuspended in SDS/PAGE loading buffer for immunoblotting. The following antibodies were used: mouse anti-Htt monoclonal antibody (4C8, Chemicon, 1:2,000); rabbit antihemagglutinin (anti-HA) epitope tag polyclonal antibody (HA.11, Covance, Princeton, NJ, 1:1,000); mouse anti-Myc epitope tag monoclonal antibody (9E10, Monoclonal Antibody Core, University of Colorado Health Sciences Center, Denver, 1:50); mouse anti-FLAG epitope tag monoclonal antibody (M2, Sigma–Aldrich, 1:2,000). The pCS2+ and pCS2+MT

Abbreviations: Htt, huntington; bHLH, basic helix–loop–helix; HAP1, Htt-associated protein 1; HD, Huntington's disease; MLK2, mixed-lineage kinase 2; HA, hemagglutinin; MT, Myc-tagged; ND, NeuroD; LZ, leucine zipper.

†To whom correspondence should be addressed. E-mail: Jackie.Lee@colorado.edu.

www.pnas.org/cgi/doi/10.1073/pnas.1133382100

vectors have been described in ref. 30. The HA-MLK2 expression plasmid has been described in ref. 31. The pCS2+HA vector was generated by cloning the fragment of pKH3 (the HA-MLK2 vector backbone) containing three copies of the HA epitope tag into the pCS2+ vector. Expression plasmids for ND, ND2, and MyoD were generated by cloning cDNA fragments corresponding to the full-length mouse proteins into the pCS2+MT or pCS2+HA vector, downstream of and in-frame with the Myc or HA epitope tag, respectively. A cDNA fragment corresponding to the full-length HAP1-B mouse protein fused at the N terminus with one copy of the FLAG epitope tag was generated by PCR and cloned into the pCS2+ vector to obtain the FL-HAP1 expression plasmid.

Alkaline Phosphatase Treatment. N2A cells were transiently transfected with the MT-ND expression plasmid, in six-well tissue culture plates. One day after transfection, N2A cells were lysed with 500 μl of ice-cold lysis buffer [50 mM Tris (pH 8.5)/150 mM sodium chloride/1% (vol/vol) Igepal CA-630 (Sigma–Aldrich)/10% (vol/vol) glycerol/Complete protease inhibitors (EDTA-free, Roche Applied Science)/1 mM magnesium chloride] and incubated on ice for 30 min. The lysate was then centrifuged at 16,000 $\times$ *g* for 30 min at 4°C, and 50 units of alkaline phosphatase (calf intestinal, Promega) was added to the supernatant, which was then incubated for 30 min at 30°C. To stop the reaction, 500 μl of 2$\times$ SDS/PAGE loading buffer was added, and an aliquot was analyzed by SDS/PAGE electrophoresis and immunoblotting.

***In Vitro* Kinase Assay.** The MBP-MLK2 expression vector was generated by cloning a cDNA fragment corresponding to amino acids 1–457 of the human MLK2 protein into the pMAL-c2 plasmid (New England Biolabs), downstream of and in-frame with the MBP affinity tag. A kinase-dead version of MBP-MLK2 (K125E) was generated by using the QuikChange site-directed mutagenesis kit (Stratagene) as directed by the manufacturer. Recombinant MBP-MLK2 proteins were purified by using the pMAL protein fusion and purification system (New England Biolabs) as directed by the manufacturer. MBP2 (New England Biolabs) consists of MBP plus the amino acids encoded by the pMAL-c2 polylinker. The immunocomplex kinase assay was performed by transfecting N2A cells with the MT-ND expression vector. One day after transfection, N2A cells were lysed with 1 ml of ice-cold 50 mM Tris (pH 7.5)/150 mM sodium chloride/1% (vol/vol) Igepal CA-630 (Sigma–Aldrich)/10% (vol/vol) glycerol/Complete protease inhibitors (EDTA-free, Roche Applied Science)/1 mM EGTA/1.5 mM magnesium chloride/phosphatase inhibitors (1 mM sodium orthovanadate/10 mM sodium fluoride/10 mM β-glycerophosphate) and incubated on ice for 30 min. The lysate was then centrifuged at 16,000 $\times$ *g* for 30 min at 4°C and the supernatant immunoprecipitated with 1–5 μg of purified antibody and 50 μl of a 50% (vol/vol) protein G-agarose slurry (Roche Applied Science). After overnight incubation at 4°C on a rotator, the protein G-agarose immunocomplex was washed twice with lysis buffer and twice with kinase buffer [25 mM Tris (pH 7.5)/100 mM sodium chloride, 10% (vol/vol) glycerol/1 mM EGTA/15 mM magnesium chloride/phosphatase inhibitors (0.1 mM sodium orthovanadate/1 mM sodium fluoride/1 mM β-glycerophosphate)]. After the final wash, the pellet was resuspended in 30 μl of kinase buffer supplemented with 5 μg of purified recombinant MBP-MLK2, 50 μM ATP, and 5 μCi of [γ-^{32}]ATP (Perkin–Elmer), and then incubated for 30 min at 30°C. To stop the reaction, 10 μl of 4$\times$ SDS/PAGE loading buffer were added and an aliquot was analyzed by SDS/PAGE electrophoresis and PhosphorImager (Molecular Dynamics) autoradiography.

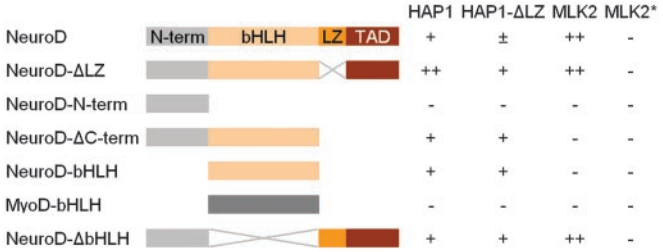

Fig. 1. ND interacts with HAP1 and MLK2 in the yeast two-hybrid system. To map the domains of interaction among ND, HAP1, and MLK2, the following deletion and point mutants were tested for interaction in the yeast two-hybrid system: ND (amino acids 73–232); ND-ΔLZ (Δ amino acids 169–190); ND-N-term (amino acids 73–97); ND-ΔC-term (amino acids 73–167); ND-bHLH (amino acids 102–154); MyoD-bHLH (mouse MyoD, amino acids 110–160); ND-ΔbHLH (Δ amino acids 102–154); mouse HAP1-A (HAP1, amino acids 247–446); HAP1ΔLZ (Δ amino acids 270–291); mouse MLK2 (corresponding to amino acids 390–460 of human MLK2); MLK2* (L426P). ++, 0–30 min β-galactosidase (β-gal) signal detection time; −, no detectable β-gal signal after 3 h.

***Xenopus* Ectopic Neurogenesis Assay.** Capped mRNA was synthesized *in vitro* by using the mMESSAGE mMACHINE SP6 kit (Ambion, Austin, TX) as directed by the manufacturer and injected at the following amounts: 100 pg of MT-ND, 1 ng of MT-MLK2, 1 ng of FL-HAP1, and 1 ng of N480–17/68Q Htt. Whole-mount immunostaining was performed as in ref. 30 by using a rabbit anti-N-CAM polyclonal antibody (32) (1:500). A cDNA fragment corresponding to the full-length human MLK2 protein fused at the N terminus with one copy of the Myc epitope tag was subcloned from the pRK5-Myc-MLK2 plasmid (33) into the pCS2+ vector to obtain the MT-MLK2 expression plasmid. A kinase-dead version of MT-MLK2 (K125E) was generated by using the QuikChange site-directed mutagenesis kit (Stratagene) as directed by the manufacturer. A cDNA fragment corresponding to amino acids 1–480 of the mouse Htt protein was subcloned from the 480–17/68 Htt plasmids (34) into the pCS2+ vector to obtain the N480–17/68Q Htt expression plasmids.

Results

ND Interacts with HAP1 and MLK2 in the Yeast Two-Hybrid System. To identify proteins that interact with ND, we performed a yeast two-hybrid screen by using ND as bait. From this screen, we isolated two proteins previously shown to interact with Htt, HAP1 and MLK2. We then used the yeast two-hybrid system to map the domain(s) of ND that interacts with HAP1 and MLK2 (Fig. 1). ND consists of: (*i*) an N-terminal domain; (*ii*) a bHLH domain; and (*iii*) a C-terminal domain containing an atypical leucine-zipper (LZ) motif and the transcriptional activation domain (TAD). Because HAP1 and MLK2 also contain a LZ motif, we examined whether deleting or inactivating the LZ motif of ND, HAP1, or MLK2 affects the interactions among these proteins. Surprisingly, deleting the LZ motif of ND (ND-ΔLZ) did not abolish its interaction with HAP1 or MLK2. Similarly, the interaction between ND and HAP1 was not abrogated by deleting the LZ motif of HAP1 (HAP1-ΔLZ). However, the interaction between ND and MLK2 was abolished by introducing a point mutation that inactivates the LZ motif of MLK2 (MLK2*). Because it has been previously shown that MLK2 functions as a dimer, and that the LZ motif of MLK2 is essential for its dimerization (35), it is possible that MLK2 interacts with ND as a dimer, and that disruption of MLK2 dimerization abolishes interaction with ND. Next, we examined the interaction of HAP1 and MLK2 with the other domains of ND. The N-terminal domain alone (ND-N-term) failed to interact with either HAP1 or MLK2. However, a deletion

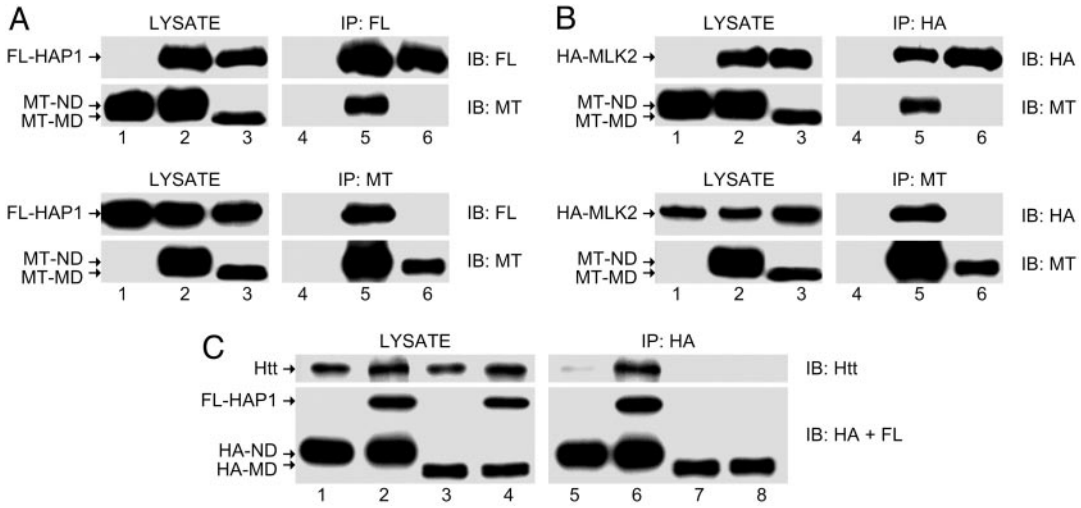

Fig. 2. ND interacts with Htt, HAP1, and MLK2 in a neuronal cellular context. (*A*) To test the interaction between HAP1 and ND, coimmunoprecipitation experiments (*Upper*) were performed by transfecting N2A cells with plasmids expressing the following proteins: Myc-tagged ND (MT-ND) alone (lanes 1 and 4); FLAG-tagged HAP1 (FL-HAP1) and MT-ND (lanes 2 and 5); FL-HAP1 and Myc-tagged MyoD (MT-MD) (lanes 3 and 6). Reciprocal coimmunoprecipitation experiments (*Lower*) were performed by transfecting N2A cells with plasmids expressing the following proteins: FL-HAP1 alone (lanes 1 and 3); MT-ND and FL-HAP1 (lanes 2 and 5); and MT-MD and FL-HAP1 (lanes 3 and 6). The amount of the corresponding proteins in the cell lysate before immunoprecipitation (*Upper* and *Lower*, lanes 1–3) was analyzed by immunoblotting with antibodies against the FLAG (FL) and Myc (MT) epitope tags. FL-HAP1 (*Upper*, lanes 4–6) and MT-ND (*Lower*, lanes 4–6) were immunoprecipitated by using an antibody against the FLAG (FL) or Myc (MT) epitope tag, respectively, and the composition of the precipitate was analyzed by immunoblotting as described above. (*B*) To test the interaction between MLK2 and ND, coimmunoprecipitation experiments were performed as described above where the plasmid expressing FL-HAP1 was replaced by the plasmid expressing HA-tagged MLK2 (HA-MLK2), and the antibody against the FLAG epitope tag was replaced by an antibody against the HA epitope tag. (*C*) To test the interaction between Htt and ND via HAP1, coimmunoprecipitation experiments were performed by transfecting N2A cells with plasmids expressing the following proteins: HA-tagged ND (HA-ND) alone (lanes 1 and 5); HA-ND and FL-HAP1 (lanes 2 and 6); HA-MD alone (lanes 3 and 7); and HA-MD and FL-HAP1 (lanes 4 and 8). The amount of the corresponding exogenous proteins and of endogenous Htt in the cell lysate before immunoprecipitation (lanes 1–4) was analyzed by immunoblotting with antibodies against the FLAG (FL) and HA epitope tags and an antibody against Htt. HA-ND (lanes 5–8) was immunoprecipitated by using an antibody against the HA epitope tag, and the composition of the precipitate was analyzed by immunoblotting as described above. Results shown are representative of at least three independent experiments. IP, immunoprecipitate; IB, immunoblot.

mutant of ND containing the N-terminal and bHLH domains (ND-ΔC-term) interacted with HAP1 but not with MLK2, indicating that the bHLH domain of ND mediates the interaction between ND and HAP1. Indeed, the bHLH domain of ND (but not that of another bHLH transcription factor MyoD) was sufficient to mediate the interaction between ND and HAP1 but not between ND and MLK2. However, a deletion mutant of ND lacking the bHLH domain (ND-ΔbHLH) still interacted with both HAP1 and MLK2, indicating that ND interacts with HAP1 not only via its bHLH domain but also via its C-terminal domain, which also mediates the interaction with MLK2.

ND Interacts with Htt, HAP1, and MLK2 in a Neuronal Cellular Context. To confirm the interaction of ND with HAP1 and MLK2 in a neuronal cellular context, we performed coimmunoprecipitation experiments (Fig. 2 *A* and *B*, respectively). Because several ND antibodies that we generated or tested were not suitable for coimmunoprecipitation from brain lysates, we transfected mouse neuroblastoma (N2A) cells with plasmids expressing epitope-tagged ND in combination with HAP1 or MLK2. We used MyoD as a control for the specificity of the interactions. Immunoprecipitation of HAP1 or MLK2 resulted in coprecipitation of ND but not of MyoD. Conversely, immunoprecipitation of ND, but not of MyoD, resulted in coprecipitation of HAP1 or MLK2. Coimmunoprecipitation experiments using ND2, a closely related member of the ND family, yielded similar results (data not shown), indicating that HAP1 and MLK2 preferentially bind to members of the ND family in a neuronal cellular context.

To test whether ND forms a complex with Htt via its interaction with HAP1, we performed double-coimmunoprecipitation experiments (Fig. 2*C*). N2A cells, which express very low levels of endogenous HAP1 (data not shown), were transfected with plasmids expressing epitope-tagged ND with or without HAP1. Immunoprecipitation of ND resulted in substantial coprecipitation of endogenous Htt only when HAP1 was coexpressed with ND. This result indicates that HAP1 can act as an adapter linking ND to Htt.

MLK2 Phosphorylates ND. Binding of MLK2 to ND suggests that ND may be a substrate for MLK2 (36). To test whether MLK2 phosphorylates ND, we examined the phosphorylation state of epitope-tagged ND in transfected N2A cells (Fig. 3*A*, lanes 1 and 2). By immunoblotting, we found that ND resolves as several bands, indicating that ND is posttranslationally modified into several isoforms. We demonstrated that this banding pattern, at least in part, is due to phosphorylation, by treating ND with alkaline phosphatase and showing that the dephosphorylated ND migrates predominantly as one band with faster mobility than untreated ND. To determine whether MLK2 leads to phosphorylation of ND *in vivo*, we transfected N2A cells with plasmids expressing epitope-tagged ND in combination with MLK2 (Fig. 3*A*, lanes 3 and 4). Cotransfection of wild-type, but not kinase-dead, MLK2 caused a shift in the mobility of ND to slower migrating forms, indicating that MLK2 leads to phosphorylation of ND *in vivo*.

Next, we performed *in vitro* kinase assay experiments by using epitope-tagged ND purified by immunoprecipitation from transfected N2A cells and purified recombinant MLK2 (Fig. 3*B*). Wild-type, but not kinase-dead, MLK2 was able to phosphorylate ND *in vitro*. Taken together, these results indicate that MLK2 phosphorylates ND. However, we found that purified recombinant ND was not a good substrate for MLK2, suggesting that phosphorylation of ND by MLK2 may require prior mod-

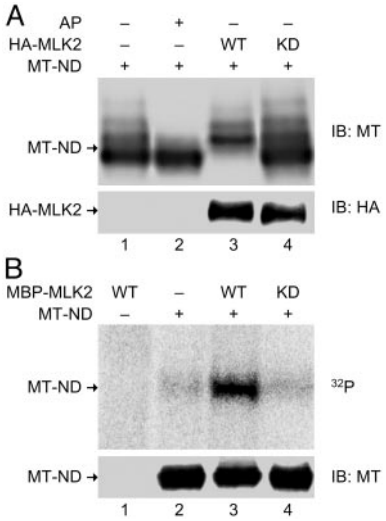

Fig. 3. MLK2 phosphorylates ND. (*A*) To examine the phosphorylation state of ND *in vivo*, immunoblotting experiments were performed by transfecting N2A cells with plasmids expressing MT-ND. The cell lysate was treated with alkaline phosphatase (AP) before immunoblotting (lane 2; lane 1 is untreated cell lysate as negative control). MT-ND was detected by immunoblotting with an antibody against the Myc epitope tag (MT, *Upper*). To test the phosphorylation of ND by MLK2 *in vivo*, immunoblotting experiments were performed by transfecting N2A cells with plasmids expressing MT-ND with either wild-type (WT) (lane 3) or kinase-dead (KD) (lane 4) HA-MLK2. MT-ND was detected by immunoblotting with an antibody against the Myc epitope tag (MT, *Upper*). To allow the resolution of the ND banding pattern, lane 3 was loaded as 20% of lane 4, in *Upper* only. The amount of HA-MLK2 in the cell lysate was analyzed by immunoblotting with an antibody against the HA epitope tag (*Lower*). (*B*) To test the phosphorylation of ND by MLK2 *in vitro*, immunocomplex kinase assay experiments were performed by transfecting N2A cells with empty vector (lane 1) or with the plasmid expressing MT-ND (lanes 2–4). MT-ND was immunoprecipitated with an antibody against the Myc epitope tag and then added to kinase reaction buffer supplemented with radiolabeled ATP and the following proteins: WT purified recombinant MLK2 (MBP-MLK2) (lane 1); MBP2 (lane 2); WT MBP-MLK2 (lane 3); KD MBP-MLK2 (lane 4). MT-ND was resolved by SDS/PAGE electrophoresis, and incorporation of radiolabeled ATP was then analyzed by PhosphorImager autoradiography (^{32}P, *Upper*). The amount of MT-ND in the precipitate was analyzed by immunoblotting with an antibody against the Myc epitope tag (MT, *Lower*). Results shown are representative of at least three independent experiments.

ification of the protein or additional cofactors that may be present in the immunoprecipitate.

Htt, HAP1, and MLK2 Stimulate ND Activity. Because phosphorylation of ND has been implicated in the regulation of ND activity (37, 38), we hypothesized that MLK2 may modulate the activity of ND. To test this hypothesis, we performed quantitative ectopic neurogenesis assay experiments. In this assay, ND was overexpressed in *Xenopus* embryos by mRNA injection, resulting in ectopic neurogenesis (1). The fraction of embryos displaying ectopic neurons served as a measure of ND activity. This assay is specific and sensitive to the activity of ND as a neurogenic transcriptional activator. Indeed, a ND mutant that cannot bind to DNA because of point mutations in the basic domain cannot induce ectopic neurogenesis. On the other hand, a ND chimera composed of the N-terminal and bHLH domains of ND fused to the potent VP16 transcriptional activation domain induced strong ectopic neurogenesis in 100% of the frog embryos (data not shown). Using this assay, we first tested whether MLK2 modulates the activity of ND (Fig. 4). Injection of MLK2 alone did not cause ectopic neurogenesis (data not shown). However, injection of wild-type, but not kinase-dead, MLK2 in combination with ND resulted in increased ectopic neurogenesis by 2-fold, indicating that MLK2 phosphorylation stimulates the activity of ND.

Next, we tested whether Htt and HAP1 affect the activity of ND and/or its activation by MLK2 (Fig. 4). Because it was not possible to synthesize full-length Htt mRNA *in vitro* given its large size, we used an N-terminal fragment of Htt corresponding to amino acids 1–480, which contains the interaction domains for both HAP1 and MLK2. Injection of Htt or HAP1 alone did not cause ectopic neurogenesis (data not shown). However, injection of ND in combination with HAP1 resulted in increased ectopic neurogenesis by 2-fold. Furthermore, HAP1 was able to facilitate the activation of ND by MLK2. Unlike HAP1 and MLK2, Htt did not affect the activity of ND. However, triple coinjection of Htt, wild-type MLK2, and ND led to a synergistic stimulation of ND activity. Quadruple coinjection of Htt, HAP1, wild-type MLK2, and ND resulted in an even stronger synergistic augmentation of ND activity such that 86% of the embryos displayed ectopic neurons as compared with 15% of the embryos injected with ND alone. Using mutant Htt instead of wild-type Htt did not significantly change the outcome of these experiments (data not shown). This result is not surprising because mutant Htt can replace the function of wild-type Htt during embryonic development (22), indicating that both wild-type and mutant Htt can support embryonic neurogenesis, which is being measured in our assay. Stimulation of ND activity was not seen when ND, Htt, and HAP1 were coinjected with kinase-dead MLK2. Taken together, these results indicate that Htt, HAP1, and MLK2 function in the same signaling pathway to activate ND.

Discussion

In summary, we have shown that Htt interacts with ND via HAP1, and that MLK2 phosphorylates and stimulates the activity of ND. We have also shown that Htt and HAP1 facilitate activation of ND by MLK2. This is a previously undescribed case of a protein kinase that physically interacts with, directly phosphorylates, and consequently activates ND. Although it is simple to postulate the role of MLK2 in the modulation of ND activity, it is not obvious how to interpret the effects of Htt and HAP1. Because Htt has no effect on ND by itself but enhances ND activity in the presence of HAP1 and MLK2, we propose that Htt, together with HAP1, may function in the cytoplasm as a scaffold for activation of ND by MLK2 (Fig. 5), in an arrangement reminiscent of the intracellular domain of transmembrane cytokine receptors in the JAK-STAT signaling pathway (39). Analogously, activation of ND correlates with its translocation from the cytoplasm to the nucleus (38).

Scaffold proteins provide binding sites for two or more components of a signaling pathway so they can be tethered in close proximity to one another (40). Indeed, the ability of a scaffold protein to bring together a kinase and its target transcription factor has been recently demonstrated in the case of JLP (41). This arrangement creates a multiprotein signaling complex that enhances the efficiency and specificity of signal transduction (42). Although our finding indicates a previously undescribed function of HTT as a scaffold for a signaling module, the ability of Htt to organize protein–protein complexes is also suggested by its primary structure (43, 44). In this regard, the role of HAP1 in bridging ND and Htt is significant. Therefore, it would be interesting to address the role of HAP1 in mediating the function of ND in the recently generated HAP1-null mice (45).

Remarkably, ND is, to our knowledge, the first example of a neuron-specific transcription factor involved in neuronal development and survival whose activity is modulated by Htt. Recently, it has been suggested that the polyglutamine expansion endows mutant Htt with a toxic gain of function that causes neurodegeneration by interfering with the activity of ubiquitous transcription regulators such as Sp1 (46) and CBP (47). However, it is not clear why such transcriptional dysregulation is toxic only to neurons. One possibility is that mutant Htt also interferes

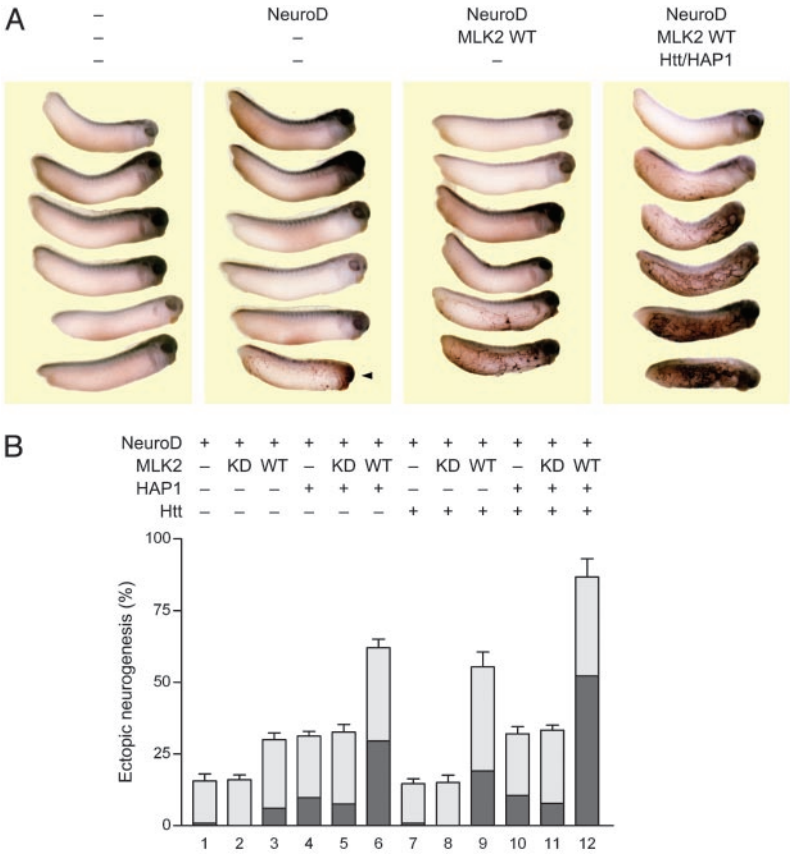

Fig. 4. Htt, HAP1, and MLK2 activate ND. (*A*) *Xenopus* embryos were injected in one blastomere at the two-cell stage with various combinations of mRNA as indicated. Formation of ectopic neurons induced by ND was visualized as dark staining at the tail-bud stage by whole-mount immunostaining with an antibody against the neuronal marker N-CAM. A limiting amount of ND mRNA was used to elicit a minimal neurogenic response when injected alone (indicated by the arrow, *Left Center*). Results shown are representative of at least three independent experiments. (*B*) Quantitation of ectopic neurogenesis induced by ND. Bar graph shows mean percent of *Xenopus* embryos with ectopic neurons ± SEM of at least three independent experiments performed as described above. Dark areas show mean percent of *Xenopus* embryos with extensive and intense ectopic neurogenesis.

with the activity of neuron-specific transcription factors (48) such as members of the ND family by, e.g., sequestering wild-type Htt in intracellular aggregates (49) and thus acting as a dominant negative factor (26). This would lead to a combinatorial and possibly more neuron-specific pattern of toxic transcriptional dysregulation. Interestingly, loss of ND2 function in the mouse brain (5) causes transcriptional down-regulation of several genes that are also reduced in animal models of HD (50). One such gene is BDNF, a neurotrophin whose expression is regulated by Htt at the transcriptional level and whose down-regulation has been implicated in the etiology of HD (25).

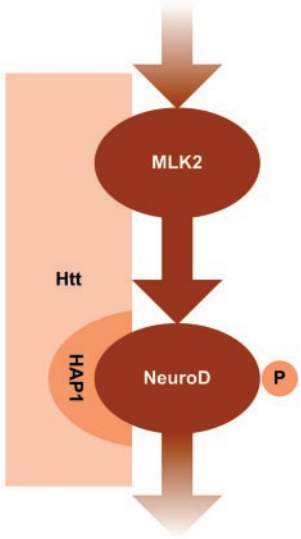

Fig. 5. Model of Htt as a scaffold for ND signaling.

Our findings are a first critical step toward understanding the molecular function of wild-type Htt in neuronal development and survival, as well as toward testing the hypothesis that loss of this function may contribute to the selective neuronal pathology of HD (28). The physiological function of Htt has eluded a coherent understanding mainly because its subcellular localization and interaction with proteins of known function implicated wild-type Htt in three apparently independent biological processes (51): (*i*) endocytic trafficking, (*ii*) signal transduction, and (*iii*) transcriptional regulation. The physical and functional interactions among Htt, HAP1, MLK2, and ND suggest that these processes are three facets of one integrated function (52–54).

We thank Alan Hall (University College London, London) and Donna Dorow (Peter MacCallum Cancer Institute, Victoria, Australia) for the pRK5-Myc-MLK2 and HA-MLK2 plasmids, respectively; and Frederic Saudou (Institut Curie, Orsay, France), Jean-Louis Mandel (Institut de Génétique et de Biologie Moléculaire et Cellulaire, Illkirch, France), and Michael Greenberg (Harvard Medical School, Boston) for the 480-17/68 Htt plasmids.

1. Lee, J. E., Hollenberg, S. M., Snider, L., Turner, D. L., Lipnick, N. & Weintraub, H. (1995) *Science* **268,** 836–844.
2. Miyata, T., Maeda, T. & Lee, J. E. (1999) *Genes Dev.* **13,** 1647–1652.
3. Liu, M., Pereira, F. A., Price, S. D., Chu, M. J., Shope, C., Himes, D., Eatock, R. A., Brownell, W. E., Lysakowski, A. & Tsai, M. J. (2000) *Genes Dev.* **14,** 2839–2854.
4. Kim, W. Y., Fritzsch, B., Serls, A., Bakel, L. A., Huang, E. J., Reichardt, L. F., Barth, D. S. & Lee, J. E. (2001) *Development (Cambridge, U.K.)* **128,** 417–426.
5. Olson, J. M., Asakura, A., Snider, L., Hawkes, R., Strand, A., Stoeck, J., Hallahan, A., Pritchard, J. & Tapscott, S. J. (2001) *Dev. Biol.* **234,** 174–187.
6. Pennesi, M. E., Cho, J. H., Yang, Z., Wu, S. H., Zhang, J., Wu, S. M. & Tsai, M. J. (2003) *J. Neurosci.* **23,** 453–461.
7. Naya, F. J., Huang, H. P., Qiu, Y., Mutoh, H., DeMayo, F. J., Leiter, A. B. & Tsai, M. J. (1997) *Genes Dev.* **11,** 2323–2334.
8. Naya, F. J., Stellrecht, C. M. & Tsai, M. J. (1995) *Genes Dev.* **9,** 1009–1019.
9. Malecki, M. T., Jhala, U. S., Antonellis, A., Fields, L., Doria, A., Orban, T., Saad, M., Warram, J. H., Montminy, M. & Krolewski, A. S. (1999) *Nat. Genet.* **23,** 323–328.
10. Poulin, G., Turgeon, B. & Drouin, J. (1997) *Mol. Cell. Biol.* **17,** 6673–6682.
11. Kim, J. W., Seghers, V., Cho, J. H., Kang, Y., Kim, S., Ryu, Y., Baek, K., Aguilar-Bryan, L., Lee, Y. D., Bryan, J., *et al.* (2002) *Mol. Endocrinol.* **16,** 1097–1107.
12. Watada, H., Scheel, D. W., Leung, J. & German, M. S. (2003) *J. Biol. Chem.* **278,** 17130–17140.
13. Bates, G., Harper, P. S. & Jones, A. L. (2002) *Huntington's Disease* (Oxford Univ. Press, Oxford), 3rd Ed.
14. Huntington's Disease Collaborative Research Group (1993) *Cell* **72,** 971–983.
15. Ross, C. A. (2002) *Neuron* **35,** 819–822.
16. Li, X. J., Li, S. H., Sharp, A. H., Nucifora, F. C., Jr., Schilling, G., Lanahan, A., Worley, P., Snyder, S. H. & Ross, C. A. (1995) *Nature* **378,** 398–402.
17. Velier, J., Kim, M., Schwarz, C., Kim, T. W., Sapp, E., Chase, K., Aronin, N. & DiFiglia, M. (1998) *Exp. Neurol.* **152,** 34–40.
18. Li, Y., Chin, L. S., Levey, A. I. & Li, L. (2002) *J. Biol. Chem.* **277,** 28212–28221.
19. Gallo, K. A. & Johnson, G. L. (2002) *Nat. Rev. Mol. Cell Biol.* **3,** 663–672.
20. Liu, Y. F., Dorow, D. & Marshall, J. (2000) *J. Biol. Chem.* **275,** 19035–19040.
21. O'Kusky, J. R., Nasir, J., Cicchetti, F., Parent, A. & Hayden, M. R. (1999) *Brain Res.* **818,** 468–479.
22. White, J. K., Auerbach, W., Duyao, M. P., Vonsattel, J. P., Gusella, J. F., Joyner, A. L. & MacDonald, M. E. (1997) *Nat. Genet.* **17,** 404–410.
23. Nasir, J., Floresco, S. B., O'Kusky, J. R., Diewert, V. M., Richman, J. M., Zeisler, J., Borowski, A., Marth, J. D., Phillips, A. G. & Hayden, M. R. (1995) *Cell* **81,** 811–823.
24. Dragatsis, I., Levine, M. S. & Zeitlin, S. (2000) *Nat. Genet.* **26,** 300–306.
25. Zuccato, C., Ciammola, A., Rigamonti, D., Leavitt, B. R., Goffredo, D., Conti, L., MacDonald, M. E., Friedlander, R. M., Silani, V., Hayden, M. R., *et al.* (2001) *Science* **293,** 493–498.
26. Cattaneo, E., Rigamonti, D., Goffredo, D., Zuccato, C., Squitieri, F. & Sipione, S. (2001) *Trends Neurosci.* **24,** 182–188.
27. Leavitt, B. R., Guttman, J. A., Hodgson, J. G., Kimel, G. H., Singaraja, R., Vogl, A. W. & Hayden, M. R. (2001) *Am. J. Hum. Genet.* **68,** 313–324.
28. Cattaneo, E. (2003) *News Physiol. Sci.* **18,** 34–37.
29. Orr, H. T. (2001) *Neuron* **31,** 875–876.
30. Turner, D. L. & Weintraub, H. (1994) *Genes Dev.* **8,** 1434–1447.
31. Phelan, D. R., Price, G., Liu, Y. F. & Dorow, D. S. (2001) *J. Biol. Chem.* **276,** 10801–10810.
32. Balak, K., Jacobson, M., Sunshine, J. & Rutishauser, U. (1987) *Dev. Biol.* **119,** 540–550.
33. Nagata, K., Puls, A., Futter, C., Aspenstrom, P., Schaefer, E., Nakata, T., Hirokawa, N. & Hall, A. (1998) *EMBO J.* **17,** 149–158.
34. Saudou, F., Finkbeiner, S., Devys, D. & Greenberg, M. E. (1998) *Cell* **95,** 55–66.
35. Leung, I. W. & Lassam, N. (1998) *J. Biol. Chem.* **273,** 32408–32415.
36. Sharrocks, A. D., Yang, S. H. & Galanis, A. (2000) *Trends Biochem. Sci.* **25,** 448–453.
37. Moore, K. B., Schneider, M. L. & Vetter, M. L. (2002) *Neuron* **34,** 183–195.
38. Petersen, H., Jensen, J., Stein, R. & Serup, P. (2002) *FEBS Lett.* **528,** 241–245.
39. O'Shea, J. J., Gadina, M. & Schreiber, R. D. (2002) *Cell* **109,** Suppl., S121–S131.
40. Smith, F. D. & Scott, J. D. (2002) *Curr. Biol.* **12,** R32–R40.
41. Lee, C. M., Onesime, D., Reddy, C. D., Dhanasekaran, N. & Reddy, E. P. (2002) *Proc. Natl. Acad. Sci. USA* **99,** 14189–14194.
42. Whitmarsh, A. J., Cavanagh, J., Tournier, C., Yasuda, J. & Davis, R. J. (1998) *Science* **281,** 1671–1674.
43. Andrade, M. A. & Bork, P. (1995) *Nat. Genet.* **11,** 115–116.
44. Takano, H. & Gusella, J. F. (2002) *BMC Neurosci.* **3,** 15.
45. Chan, E. Y., Nasir, J., Gutekunst, C. A., Coleman, S., Maclean, A., Maas, A., Metzler, M., Gertsenstein, M., Ross, C. A., Nagy, A., *et al.* (2002) *Hum. Mol. Genet.* **11,** 945–959.
46. Dunah, A. W., Jeong, H., Griffin, A., Kim, Y. M., Standaert, D. G., Hersch, S. M., Mouradian, M. M., Young, A. B., Tanese, N. & Krainc, D. (2002) *Science* **296,** 2238–2243.
47. Nucifora, F. C., Jr., Sasaki, M., Peters, M. F., Huang, H., Cooper, J. K., Yamada, M., Takahashi, H., Tsuji, S., Troncoso, J., Dawson, V. L., *et al.* (2001) *Science* **291,** 2423–2428.
48. La Spada, A. R., Fu, Y. H., Sopher, B. L., Libby, R. T., Wang, X., Li, L. Y., Einum, D. D., Huang, J., Possin, D. E., Smith, A. C., *et al.* (2001) *Neuron* **31,** 913–927.
49. Dyer, R. B. & McMurray, C. T. (2001) *Nat. Genet.* **29,** 270–278.
50. Luthi-Carter, R., Hanson, S. A., Strand, A. D., Bergstrom, D. A., Chun, W., Peters, N. L., Woods, A. M., Chan, E. Y., Kooperberg, C., Krainc, D., *et al.* (2002) *Hum. Mol. Genet.* **11,** 1911–1926.
51. Sipione, S. & Cattaneo, E. (2001) *Mol. Neurobiol.* **23,** 21–51.
52. Verhey, K. J., Meyer, D., Deehan, R., Blenis, J., Schnapp, B. J., Rapoport, T. A. & Margolis, B. (2001) *J. Cell Biol.* **152,** 959–970.
53. Kholodenko, B. N. (2002) *Trends Cell Biol.* **12,** 173–177.
54. Sorkin, A. & Von Zastrow, M. (2002) *Nat. Rev. Mol. Cell Biol.* **3,** 600–614.