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DYRK1A regulates Hap1–Dcaf7/WDR68 binding with implication for delayed growth in Down syndrome

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Huntingtin-associated protein 1 (Hap1) is a cytoplasmic protein highly expressed in neurons of the CNS (1, 2). Hap1 was initially recognized for its binding to polyglutamine-expanded huntingtin (Htt), the protein responsible for Huntington’s disease (3). Because Hap1 is expressed at various levels in different types of neurons (1, 4–8), it is likely involved in cell type-specific functions of the brain. For example, Hap1 is abundantly expressed in the hypothalamus, and its expression in the hypothalamus influences the central control of feeding (9); as a direct result, mice lacking Hap1 are malnourished and die in the early postnatal period (5, 10, 11). The function of Hap1 is also age dependent and appears to be important for postnatal neurogenesis in the paraventricular nuclei, the lateral hypothalamus (5, 11), and the dentate gyrus of the hippocampus (7).

The specific function of Hap1 in the hypothalamus may be associated with unique cytoplasmic structures called “stigmoid bodies” (SBs), which are formed by Hap1 in the hypothalamus (12, 13) and appear to be non–membrane-bound, ovoid to circular in shape, and 0.5–3 μm in diameter (14, 15). Widely regarded as a determinant marker for SBs, Hap1 is capable of sequestering several proteins associated with unique cytoplasmic structures of the brain. For example, Ahi1, anodyne receptor, TDP-43, and ataxin-3 (6, 16–18). However, in vivo evidence for such sequestration exists only for Ahi1, which is responsible for specific forms of Joubert syndrome. Other proteins, such as SorLA/LR11, sortilin, 5-HT7 receptor, and 14-3-3, are also reported to be associated with SBs (19–21). Although SBs are abundant in the hypothalamus, their functions remain elusive, despite speculation that they might be involved in sex-steroid maturation (15). We believe that identifying new protein components of the SBs will help shed light on the specific function of Hap1 in the hypothalamus.

In the current study, we started out by developing a strategy to allow the identification of proteins interacting with Hap1 in hypothalamic SBs. Through mass spectrometry analysis followed by validation of candidate proteins, we identified DDB1 and CUL4 associated factor 7 (Dcaf7)/WD40 repeat 68 (WDR68) as a protein that interacts with Hap1 and a component of the SB. Dcaf7 contains WD40-repeat (WD) domains, is highly conserved from animals to plants, and is involved in a variety of cellular processes. It binds DYRK1A, a dual-specificity tyrosine phosphorylation-regulated kinase that is encoded by a gene located on chromosome 21 and is overexpressed in Down syndrome (DS) (22). DYRK1A is a major candidate gene in producing features of DS, and transgenic mice overexpressing DYRK1A show cognitive and behavioral alterations similar to those in persons with DS (23, 24). Also, recent studies show that administration of epigallocatechin-3-gallate (EGCG), which antagonizes DYRK1A, can improve the cognitive function of individuals with DS (25, 26).

We found that Hap1 controls the protein level of Dcaf7 and inhibits its nuclear translocation. More importantly, Hap1 and DYRK1A competitively bind Dcaf7 in the cytoplasm, and DYRK1A overexpression in the hypothalamus of DS mice leads to a decreased association between Hap1 and Dcaf7. Furthermore, we found that mice injected in the hypothalamus with Adeno-associated viruses (AAV) expressing DYRK1A gained less body weight during the postnatal period; this result could explain, in part, the growth retardation phenotype in DS. Thus, our work offers insights into the mechanism underlying the postnatal growth retardation of DS mice.

Significance

Postnatal growth retardation is a Down syndrome (DS) phenotype, but its mechanism is unknown. Recent studies have shown that Huntingtin-associated protein 1 (Hap1) is important for the postnatal growth of mice. Here we report that Hap1 binds DDB1- and CUL4-associated factor 7 (Dcaf7) competitively in the cytoplasm with dual-specificity tyrosine phosphorylation-regulated kinase 1A (DYRK1A), a protein implicated in DS. Loss of Hap1 in the hypothalamus, which can impair postnatal growth, is associated with increased levels of DYRK1A. Transgenic DS mice overexpressing DYRK1A show reduced Hap1–Dcaf7 association in the hypothalamus. Furthermore, the overexpression of DYRK1A in the hypothalamus led to delayed growth in postnatal mice, suggesting that DYRK1A regulates the Hap1–Dcaf7 interaction and postnatal growth and that targeting Hap1 or Dcaf7 may ameliorate growth retardation in DS.

Author contributions: S.L. and X.-J.L. designed research; J.X., S.Y., N.X., and M.A.G. performed research; R.H.R. contributed new reagents/analytical tools; J.X. analyzed data; and J.X., S.L., and X.-J.L. wrote the paper.

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retardation in DS and also opens up the possibility of targeting Hap1 or Dcaf7 as a treatment for DS.

Results

A Strategy to Enrich SBs from Mouse Brain. Because the SB is a Hap1-containing nonmembranous cytoplasmic structure (15) and because transfected Hap1A, one of the two murine Hap1 isoforms, can initiate the formation of this structure in HEK293 cells (12), we set out to see whether we could enrich SBs using the detergent Nonidet P-40. With increasing ratios of Hap1A to Hap1B transfected into HEK293 cells, we found more Hap1 was retained in the Nonidet P-40–insoluble (P) fraction (Fig. L4), indicating that the detergent-insoluble pellet indeed contained inclusions formed by Hap1A. To test the applicability of this strategy in vivo, we analyzed the relative abundance of Hap1 in Nonidet P-40–soluble (S) and –insoluble (P) fractions from mouse hippocampus, cortex, striatum, and hypothalamus. The highest P/S ratio of Hap1 was seen in the hypothalamus (Fig. 1B), in agreement with the finding that SBs are more abundant in the hypothalamus than in other tissues (5, 10, 14). Thus, these results showed that the use of a detergent-based strategy to enrich SBs in mouse brain was feasible.

Heterozygous Hap1−KO (Het) mice are suggested to have fewer Hap1-containing SBs (10). Our staining also showed a decrease in the number of Hap1-containing SBs in the hypothalamus of Het mouse brain (Fig. 1C). Next, we used a detergent-based strategy to isolate the SB-enriched fraction from mouse brain (excluding regions that express a low amount of Hap1) with help from a previous protocol (Fig. 1D) (27). Compared with Het control mice, WT mice had significantly more Hap1 in the P2 fraction, even though there was no obvious difference in soluble Hap1 level in the two groups (Fig. 1E); our results support an enrichment of Hap1-containing SBs in the P2 fraction in the hypothalamus of WT mice. Notably, the S2 fraction after 1% Nonidet P-40 treatment contained very little Hap1, suggesting that the P1 fraction is already enriched in Hap1-containing SBs that are resistant to further Nonidet P-40 extraction. Thus, the P1 fraction was used to identify Hap1 partners in our simplified strategy 2 (Fig. 1F). Indeed, the enrichment of Hap1-containing SBs in the P1 and P2 fractions was confirmed by staining Hap1 on a glass slide loaded with these fractions (Fig. S1). After resuspension in PBS and Triton X-100 (PBST), the P1 fraction was subjected to immunoprecipitation (IP) by a monoclonal antibody against Hap1. The efficiency of IP was tested via Western blotting, in which high levels of Hap1 and Ahi1, a protein that is known to bind tightly to Hap1 (6), were found in the WT sample as compared with the Het sample (Fig. 1G). With these steps we successfully developed a protocol to isolate the SB-enriched fraction, which can be used to identify novel SB-associated proteins, from mouse brain.

Dcaf7 Interacts with Hap1 and Is a Component of the SB. Next, we ran the Hap1 immunoprecipitants from WT and Het mice on a gel and performed silver staining. By comparing the banding of WT and Het samples, we identified one prominent band, A, and another weak band, B, that were more enriched in the WT sample, beyond the known bands for Hap1 and Ahi1 (Fig. 2A). The protein constitutions of the two bands were uncovered by mass spectrometry analysis (Fig. 2B). Two major proteins—a growth cone-associated protein (Gap43) (16) and a highly conserved WD40 repeat-containing scaffolding protein important for craniofacial development, Dcaf7/WDR68 (28)—were found in band A, whereas five isoforms of 14-3-3 protein were detected in band B. Because an interaction between 14-3-3 protein and Hap1 had been reported previously (20), we focused on verifying the interactions of Hap1 with the other two proteins, Gap43 and Dcaf7.

Western blotting clearly showed that Dcaf7 is specifically associated with Hap1 in the P1 (strategy 2; Fig. 1F) and P2 (strategy 1; Fig. 1D) fractions, whereas Gap43, although more enriched in the WT Hap1 immunoprecipitant, was pulled down much less efficiently by Hap1 antibody, likely in a nonspecific fashion because the control IgG also could pull down a comparable amount of Gap43 from the WT sample (Fig. 2 C and D). We therefore focused on the interaction between Dcaf7 and Hap1, which was found to exist not only in detergent-insoluble P fractions but also in the soluble S1 fraction in which Hap1 also binds tightly with Ahi1 (Fig. 2E). To confirm that Dcaf7 is indeed localized in the SB, we performed communostaining of Hap1 and Dcaf7 in sections of WT mouse hypothalamus and found both proteins were present in SBs (Fig. 2F). In Het hypothalamus, Dcaf7 appears largely diffuse and does not form puncta by itself because of the loss of Hap1 (Fig. S2), indicating that Hap1 has a central role in recruiting different components of the SB. Indeed, we saw more abundant Dcaf7-containing SBs in the hypothalamus than in the cortex (Fig. 2G), as is consistent with the greater abundance of Hap1 in the hypothalamus (Fig. 1B). Overall, we identified Dcaf7 as an Hap1-interacting protein and a component of the SB.

Binding of Hap1 Inhibits the Nuclear Translocation of Dcaf7. Dcaf7 localization to be more nuclear in the cortex appears to be more nuclear in the cortex than in the hypothalamus (Fig. 2G), raising the possibility that Hap1 may regulate the subcellular distribution of Dcaf7. To test this idea, we transfected plasmid Dcaf7-HA into HEK293 cells that do not express Hap1. When transfected alone, Dcaf7 exhibited a more nuclear localization; however, when
cotransfected with either Hap1A or Hap1B, Dcaf7 became noticeably more cytoplasmic (Fig. 3A). Transfection of Hap1A or Hap1B, but not Ah1, also redirected endogenous Dcaf7 into the cytoplasm (Fig. S3A), suggesting that Hap1 alone is able to direct the cytoplasmic distribution of Dac7. Subcellular fractionation of HEK293 cells and Western blotting using MAPK/ERK kinase 1/2 (MEK1/2) as a cytoplasmic marker and lysine-specific histone deethylase 1 (LSD1) as a nuclear marker validated a more cytoplasmic distribution of endogenous Dcaf7 in the presence of transfected Hap1B (Fig. 3B). Using additional cultured cell lines, we also showed that Hap1A was able to sequester Dcaf7 into cytoplasmic inclusions resembling SBs in vivo in both HEK293 and PC12 cells (Fig. 3A and Fig. S3).

If Hap1 indeed regulates the subcellular distribution of Dcaf7, we reasoned that we should see a perturbed distribution pattern of Dcaf7 in Hap1-KO mouse brain. Therefore, we conducted fractionation of P2 WT and Hap1-KO mouse brains and found that the loss of Hap1 led to more nuclear translocation of Dcaf7 (Fig. 3C). Because the mixed tissue types used for Western blotting express Hap1 at different levels, we used immunocytochemistry to examine Dcaf7 distribution in the hypothalamus, which is enriched in Hap1, and observed a shift in Dac7 localization leaning toward the nucleus, as well as a loss of cytoplasmic Dcaf7 puncta, when Hap1 is depleted in KO mice (Fig. 3D). Similarly, in the brainstem, which also expresses Hap1 at a high level, loss of Hap1 results in the nuclear distribution of Dac7 in neuronal cells (Fig. S4).

**Hap1 Stabilizes the Protein Level of Dcaf7 by Inhibiting Its Proteasome Degradation.** Because Hap1 regulates the protein level of its most prominent binding partner, Ah1 (6), we next examined whether Hap1 may also play a role in Dcaf7 protein turnover. In Hap1-KO mouse hypothalamus, Dcaf7 was reduced drastically, by more than 50% (Fig. 4A). No difference in Dcaf7 was seen in Hap1-KO cortex, in which very little Hap1 was expressed (Fig. S5A). In 15-d-old mice, ...
or 1-y-old conditional Hap1-KO mice in which Hap1 was deleted via tamoxifen injection at postnatal day 1 or 2 mo of age, decreased levels of Dcaf7 in the hypothalamus were also observed (Fig. 4B). However, Dcaf7 mRNA levels remained unaltered in KO hypothalamus (Fig. 4C), suggesting that Hap1 stabilizes Dcaf7 at the protein level. In addition, knocking down Hap1 in PC12 cells via siRNA also induced a dramatic reduction in the Dcaf7 protein level (Fig. 4D).

Next, we transfected Hap1A, Hap1B, or Ahi1 into HEK239 cells and found that Hap1, but not Ahi1, increased the protein level of Dcaf7 (Fig. 4E). Both Hap1A and Hap1B extended the half-life of endogenous Dcaf7 in HEK293 cells (Fig. S5B). To find out which pathway was primarily used to degrade Dcaf7, we treated HEK293 cells with either the proteasome inhibitor MG132 or the autophagy inhibitor bafilomycin A1 (BFA). Only MG132 treatment significantly stabilized the level of Dcaf7, suggesting that Dcaf7 is degraded mainly via the ubiquitin-proteasome system (UPS) (Fig. S5C). To examine whether Hap1 inhibits the proteasome degradation of Dcaf7, we compared the levels of nuclear export signal (NES)-tagged Dcaf7-HA after MG132 treatment with or without Hap1B cotransfection. We used NES-Dcaf7-HA so that we would assess only the degradation of Dcaf7 in the cytoplasm where Hap1 localizes. The result demonstrated that the proteasome degradation of Dcaf7 over the course of 16 h was remarkably decreased in the presence of Hap1B (Fig. 4F). In addition, Hap1B also reduced the polyubiquitination of immunoprecipitated NES-Dcaf7-HA (Fig. 4G). Thus, we concluded that Hap1 not only sequesters but also stabilizes Dcaf7 in the cytoplasm by inhibiting its degradation by the UPS. Because Hap1 can protect internalized receptors from lysosomal degradation (11, 29–31), Hap1 may stabilize its binding partners from subsequent degradation through either the lysosome or UPS.

**Hap1 Competes for Dcaf7 Binding with DYRK1A, Levels of Which Are Increased in Hap1-KO Mouse Brains.** We know that Dcaf7 also binds DYRK1A (32), a kinase that phosphorylates a wide range of proteins involved in metabolism, synaptic function, and neurodegeneration (33). Of greatest interest, DYRK1A maps to human chromosome 21 and perhaps is implicated in DS (34–36). We were interested in whether Hap1 and DYRK1A interact with Dcaf7 competitively or synergistically. First, however, we wanted to examine the subcellular localization of DYRK1A, because studies have demonstrated both cytoplasmic and nuclear distribution...
of endogenous DYRK1A in neuronal cells, whereas ectopically expressed DYRK1A is found mainly in the nucleus (37–41). We found via subcellular fractionation and immunostaining that endogenous DYRK1A is localized predominantly in the cytoplasm in mouse brain in vivo and in cultured HEK293 and PC12 cells (Fig. 5A and B and Fig. S6 A and B). Next, we evaluated the interaction between Dcaf7 and DYRK1A in HEK293 cells with or without Hap1. It is known that both Hap1A and Hap1B form heterodimers to interact with other proteins. Because Hap1A transfection leads to the formation of insoluble puncta in transfected cells, whereas Hap1B alone remains soluble (12), we used Hap1B transfection to examine the interaction of soluble Hap1 with other proteins. The result indicated attenuated binding between Dcaf7 and DYRK1A in the presence of Hap1B, suggesting competition between Hap1 and DYRK1A for Dcaf7 binding (Fig. 5C). Because Hap1 is strictly a cytoplasmic protein in neurons, this competition most likely happens in the neuronal cytoplasm; thus, it is reasonable to assume that the association of Dcaf7 and DYRK1A may be enhanced in the Hap1-KO mouse brain. Indeed, we saw a potentiated Dcaf7–DYRK1A interaction in Hap1-KO brain, despite a decrease in the protein level of Dcaf7 (Fig. 5D).

Surprisingly, when protein levels of DYRK1A were assessed, we saw an increase of about 70% in Hap1-KO mouse brains compared with WT controls (Fig. 5E). The up-regulation occurs...
posttranscriptionally, because mRNA levels of DYRK1A were comparable in WT and KO mice (Fig. 5F). In conditional Hap1-KO mice in which Hap1 had been depleted via postnatal tamoxifen injection (11), increased levels of DYRK1A were also observed (Fig. S6C). Because of the enhanced association between Dcaf7 and DYRK1A in Hap1-KO brain, we hypothesized that the binding of Dcaf7 might be important for DYRK1A stability. To test this hypothesis without confounding factors, such as nuclear transportation and changes in the level of Dcaf7 protein, we expressed equal amounts of NES-Dcaf7 in HEK293 cells with or without transfected Hap1B and measured the levels of endogenous DYRK1A. DYRK1A levels were reduced in groups coexpressing Hap1B, probably because a portion of NES-Dcaf7 was bound to Hap1B (Fig. 5G).

Overexpression of DYRK1A Impairs Hap1–Dcaf7 Binding and Causes Body Weight Reduction in Mice. The increased DYRK1A level in the Hap1-KO mouse brain was particularly interesting because DYRK1A also is overexpressed in the brains of individuals with DS, and this overexpression may explain some of the neuropathological traits of this syndrome (22, 42). To validate the competition between Hap1 and DYRK1A for binding with Dcaf7 in a different and more disease-relevant model, we examined the association of Hap1 and Dcaf7 in a DS mouse model, Ts65Dn, which carries a partial trisomy of mouse chromosome 21, including the DYRK1A gene (43, 44). In the hypothalamus of Ts65Dn mice overexpressing DYRK1A, the interaction between Hap1 and Dcaf7 was dramatically diminished (Fig. 6A). Ts65Dn mice exhibit neurodevelopmental delay and reduced body weight similar to that seen in individuals with DS (45, 46). Because Hap1-KO mice suffer from growth retardation (5, 10), we proposed that in DS and its mouse models elevated DYRK1A pulls Dcaf7 away from Hap1, thereby influencing the normal function of Hap1 and thus contributing to the growth defect. To test this notion, we injected AAV-expressing DYRK1A-Myc into mouse hypothalamus (a major brain region in which Hap1 exerts its feeding-regulation function) at postnatal day 1 or 2 (9). The expression of the viral vector could be verified via immunostaining of the

Fig. 5. DYRK1A and Hap1 compete for Dcaf7 binding in the cytoplasm, and the protein level of DYRK1A is increased in Hap1-KO mouse brains. (A) Fractionation of postnatal day 2 mouse brains reveals a preponderantly cytoplasmic distribution of DYRK1A. T, total; C, cytoplasmic; N, nuclear. (B) Immunostaining of DYRK1A in postnatal day 2 mouse hypothalamus also demonstrates cytoplasmic localization of DYRK1A. (Scale bar: 10 μm.) (C) Hap1B expression significantly reduces the interaction between DYRK1A and Dcaf7 in HEK293 cells. Ratios were normalized to control level. n = 3 each. (D) The association between DYRK1A and Dcaf7 is dramatically enhanced in Hap1-KO mouse brains. Ratios were normalized to the WT level. n = 3 per group. (E) The protein level of DYRK1A is increased in postnatal day 1 Hap1-KO hypothalamus. Ratios were normalized to WT level. n = 4 each. (F) Real-time RT-PCR found no difference in DYRK1A mRNA levels in postnatal day 2 WT and Hap1-KO mouse hypothalami. n = 4 per group. (G) The expression of transfected NES-Dcaf7 was adjusted at similar levels in groups with or without Hap1B cotransfection in HEK293 cells. The presence of Hap1B reduces the level of endogenous DYRK1A. Ratios were normalized to the NES-Dcaf7-only group. n = 3 per group. *P < 0.05; **P < 0.01; ***P < 0.001, n.s., not significant.
Myc tag (Fig. 6B). We then measured the body weight of injected mice and found weight gain was significantly reduced starting from postnatal days 14–17 in mice injected with AAV-DYRK1A as compared with the mice injected with the AAV control (Fig. 6C). Because recombinant AAV does not integrate into the host cell genome, the overexpression of DYRK1A in the injected region was not sustained over a long time. Also, the rapid and dramatic increase in brain volume and neurogenesis that occur during postnatal development (47, 48) could dilute the effect of AAV-transduced neurons.

Fig. 6. Overexpression of DYRK1A in mouse hypothalamus reduces the Hap1–dcaf7 interaction and leads to reduced body weight gain. (A) IP of Hap1 in the DYRK1A-overexpressing DS mouse model. Ts65Dn hypothalamus reveals a decreased association of Hap1 and Dcaf7. (B) Immunostaining of Myc in postnatal day 20 WT mouse hypothalamus that had been injected with AAV-DYRK1A-Myc or AAV-control at postnatal day 2. Enlarged images of the boxes are shown below. (Scale bars: 15 μm.) (C) Body weight recording indicates that DYRK1A overexpression in the hypothalamus reduced the postnatal weight gain of WT mice. n = 11–20. *P < 0.05; **P < 0.01.
The importance of the Hap1–Dcaf7–DYRK1A interaction in postnatal growth can be tested further by examining the cellular function related to this interaction. Our data have shown that loss of Hap1 can lead to enhanced interaction between Dcaf7 and DYRK1A, resulting in an increase in DYRK1A. It has been reported that increased DYRK1A can affect cell-cycle regulation and neuronal differentiation by reducing cyclin D1 and the cyclin-dependent kinase inhibitor p21 (49, 50), two important proteins that promote neuronal differentiation (50–54). Thus, we isolated hypothalamic tissues from WT and Hap1-KO postnatal pups and performed Western blotting with anti-cyclin D1 and anti-p21. We found that cyclin D1 and p21 were indeed decreased in the Hap1-KO hypothalamic tissues (Fig. 7 A and B). This finding also supports the role of altered Hap1–Dcaf7–DYRK1A interactions in the delayed growth of postnatal mice. Based on these findings, we propose that Dcaf7 binds both Hap1 and DYRK1A in the cytoplasm. The Hap1–Dcaf7 interaction may be critical for postnatal growth, and the loss of Hap1 in KO mice impairs postnatal growth. In DS, overexpressed DYRK1A reduces the Hap1–Dcaf7 interaction in the hypothalamus, also affecting postnatal growth (Fig. 7 C).

Discussion

Our previous findings and those of others have shown that Hap1 is highly expressed in the hypothalamus and that the loss of Hap1 affects postnatal growth (5, 10, 11). However, the mechanisms behind Hap1’s function in the hypothalamus and the relevance to human diseases of the defective postnatal growth mediated by the loss of Hap1 remain unclear. The findings in our current study suggest that the interaction of Hap1 with Dcaf7 in the hypothalamus is important for postnatal growth and that overexpressed DYRK1A could affect this interaction, thereby affecting postnatal growth in DS.

The interaction between Dcaf7 and Hap1 is strongly supported by their coexistence in hypothalamic SBs and their communoprecipitation and also by the regulation of Dcaf7 stability at the protein level by Hap1. We show that Hap1 not only stabilizes the protein level of Dcaf7 but also sequesters Dcaf7 from entering the nucleus. In the Hap1-KO mouse hypothalamus, Dcaf7 is downregulated, and its distribution becomes more nuclear. Whether its loss of function in the cytoplasm or gain of function in the nucleus promotes the deleterious effects of Hap1 deletion is unknown. Characterization of Dcaf7 function in mammals is limited, so we can only make inferences from studies in other organisms or cell cultures. For example, in zebrafish (Danio rerio), nuclear localization of Dcaf7 is required for craniofacial development (47, 48). It is logical to think that the subcellular localization of Dcaf7 may depend largely on the expression profiles of its cell type-specific interacting proteins as well as on the timing of its expression during development. Thus, it would be interesting to investigate whether the accumulation of Dcaf7 in the nuclei of Hap1-null hypothalamic neurons might be detrimental to the cell, e.g., by causing transcriptional dysregulation. Moreover, knocking down Dcaf7 in COS7 cells has been reported to induce apoptosis (32). Thus, future studies are needed to investigate the effects of Dcaf7 ablation on Hap1 function, SB formation, neuronal survival, and mouse behavior.

The more important finding in our study is that DYRK1A can regulate the interaction of Dcaf7 and Hap1. The interaction and relative abundance of Dcaf7 and DYRK1A are known to influence the localization of these two proteins (32, 48). Because craniofacial maldevelopment is a characteristic common to all individuals with DS (55), and the subcellular distribution of DYRK1A varies in different species and cell types, it is possible that DYRK1A may localize predominantly to the cytoplasm of...
cranial neural crest cells, and its overexpression in DS could cause more Dcaf7 to be retained in the cytoplasm, leading to impaired signaling in craniofacial development.

Our study highlights a physiological balance between Hap1–Dcaf7 and DYRK1A–Dcaf7 interactions and the perturbation of this balance in DS. Because we have demonstrated that both Hap1 and DYRK1A localize primarily to the cytoplasm of mouse hypothalamic neurons, where they compete for Dcaf7 binding, changes in the expression levels of either protein would disturb this balance and likely cause harm to neurons. DYRK1A levels are increased in individuals with DS; our findings indicate that this increase should tip the balance toward enhanced DYRK1A–Dcaf7 binding and weakened Hap1–Dcaf7 binding. As a result, the function of Hap1 may be attenuated.

We must point out that the wide range of symptoms and behavioral changes in DS are likely caused by the abnormal expression of a large array of genes and their interactions resulting from an extra copy of chromosome 21. DYRK1A is a major candidate gene for DS and likely is responsible for selective DS phenotypes. To look for DS symptoms that could be related to the regulation of DYRK1A on the Dcaf7–Hap1 interaction, we focused on growth retardation, which is a cardinal feature of DS (56) and is the most noticeable phenotype in Hap1–KO mice (5, 10). Both individuals with DS and the Hap1–KO mice exhibit impaired growth velocity after birth; the difference, however, is that in DS, growth velocity is most reduced between 6 mo and 3 y of age (56, 57), whereas Hap1–KO mice are born at normal weight but display severe failure in body weight gain shortly after birth (5, 10). These differences may reflect species differences in terms of maturation and life span to a certain extent, but are more likely explained by the differential consequences of partial loss of Hap1 function and a complete elimination of Hap1. The interactions of Hap1 with other proteins may mediate age-dependent functions, because loss of Hap1 at different ages causes completely different phenotypes (7). Also, triplication of an entire chromosome 21 or segment of this chromosome could affect multiple genes involved in growth control, so that the growth retardation in DS may be mediated by a combination of mechanisms.

The generation and characterization of different mouse models of DS may shed light on the causative role of dosage-sensitive genes in distinct DS phenotypes and aid in therapeutic development for DS (58). The most widely used DS mouse model, Ts65Dn mice, are trisomic for more than half of the mouse orthologs of genes on human chromosome 21 (59), whereas Ts1Cje mice carry a smaller segmental triplication included in Ts65Dn trisomy (60). That these two models display DS-characteristic growth retardation suggests that certain genes within the triplicated region are responsible, at least in part, for the phenotype (45, 46, 61). DYRK1A transgenic (Tg) mouse models were also made in an effort to reveal its role in DS. Cognitive deficits were noted in two models overexpressing DYRK1A, one under the sheep metallothionein 1a (sMT-1a) promoter (24) and the other in a BAC under the human DYRK1A promoter (23). Although original reports of these two models did not mention changes in body weight, a recent study showed that DYRK1A BAC Tg mice are lean and resistant to diet-induced obesity (62). The trend of decreased body weight starts from the earliest time point measured, i.e., 6 wk after birth. Because it is unclear how transgenic DYRK1A is expressed in the hypothalamus in these mouse models, it would be important to assess the phenotypes of mice that express a high level of DYRK1A in the hypothalamus. To this end, we performed a viral injection of DYRK1A into newborn mouse hypothalami and saw a reduction in weight gain in the injected mice. Although this reduction is consistent with delayed postnatal growth in DS, it is less severe than in Hap1–KO mice and individuals with DS. It is possible that viral vector injection allows only restricted and temporal expression of transgenic DYRK1A. Another possibility is that overexpression of other genes in chromosome 21 in DS may be required to stabilize a high level of DYRK1A in the hypothalamus. Furthermore, because of the difficulty in determining feeding behavior and metabolic functions of the limited number of pups injected postnatally with AAV-DYRK1A-Myc, it remains to be investigated whether the reduced body weight in these pups is caused by alterations in feeding activities or by metabolic changes, either of which could result from the dysfunction of hypothalamic neurons.

Because the function of DYRK1A may vary in different brain regions, we believe that hypothalamic DYRK1A is important for normal body growth. We also propose that diminished Hap1–Dcaf7 interaction is a likely mechanism for DYRK1A-mediated growth retardation in DS. Downregulating DYRK1A levels might not be an ideal solution, because DYRK1A acts on a large number of substrate proteins, including transcription factors, splicing factors, and synaptic proteins (42). If the level of Hap1–Dcaf7 interaction indeed mediates the function of Hap1 in food intake, enhancement of this interaction by up-regulating either Hap1 or Dcaf7 could provide promising therapeutic options for treating stunted growth in DS and other growth disorders.

**Materials and Methods**

Mice were housed in the Division of Animal Resources at Emory University on a 12-h light/dark cycle with lights on at 7 AM and lights off at 7 PM. All procedures were approved by the Animal Care and Use Committee at Emory University (2002557) (X.-J.L.) or The Johns Hopkins University (M015M347) (R.H.R.). Generation of germline Hap1–KO mice and tamoxifen-inducible Cre-ERfloxed-Hap1 mice was described previously (5, 11). Brain tissues of Ts65Dn mice were provided by R.H.R. Additional information is provided in SI Materials and Methods.

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