

**Figure 2.** Direct protein-protein interaction by HuD and active Akt1. (A) RNA-independent interaction between HuD and Akt1. Purified FLAG-Akt1 derived from extracts treated with benzonase was incubated with GST-HuD or GST. GST pull-downs were examined for copurification of Akt1 by IB (upper panel) and for pull-down efficiency by CBB staining (lower panel). (B) and (C) RNA-independent interaction between HuD and active Akt1. Recombinant active or inactive Akt1 was incubated with GST-HuD or GST. (C) Active Akt1 and GST-HuD were incubated with or without lambda protein phosphatase, pulled down with glutathione-Sepharose beads and analyzed by IB (upper panel). One of 10 inputs was analyzed by IB for total and phospho-Akt1 levels (middle and lower panel of Figure 2C).

recombinant active and inactive Akt1 and performed GST pull-down assays using purified recombinant GST-HuD and active or inactive Akt1. Interestingly, we find that the phosphorylated form of Akt1 (active Akt1) interacts with HuD, as active, but not inactive Akt1 copurifies with GST-HuD (Figure 2B). To further confirm the interaction between HuD and active Akt1, we treated the samples with lambda protein phosphatase before the pull-down assays, which lead as expected to a strong reduction of phosphorylated Akt1 within the input samples and in line with the data shown above to a strong reduction of Akt1 in GST pull-down eluates (Figure 2C). Thus, we conclude that HuD binds directly and specifically to active/phosphorylated Akt1. In good agreement with these data, we also find that HuD colocalizes with active Akt1 in PC12 cells (Supplementary Figure S1).

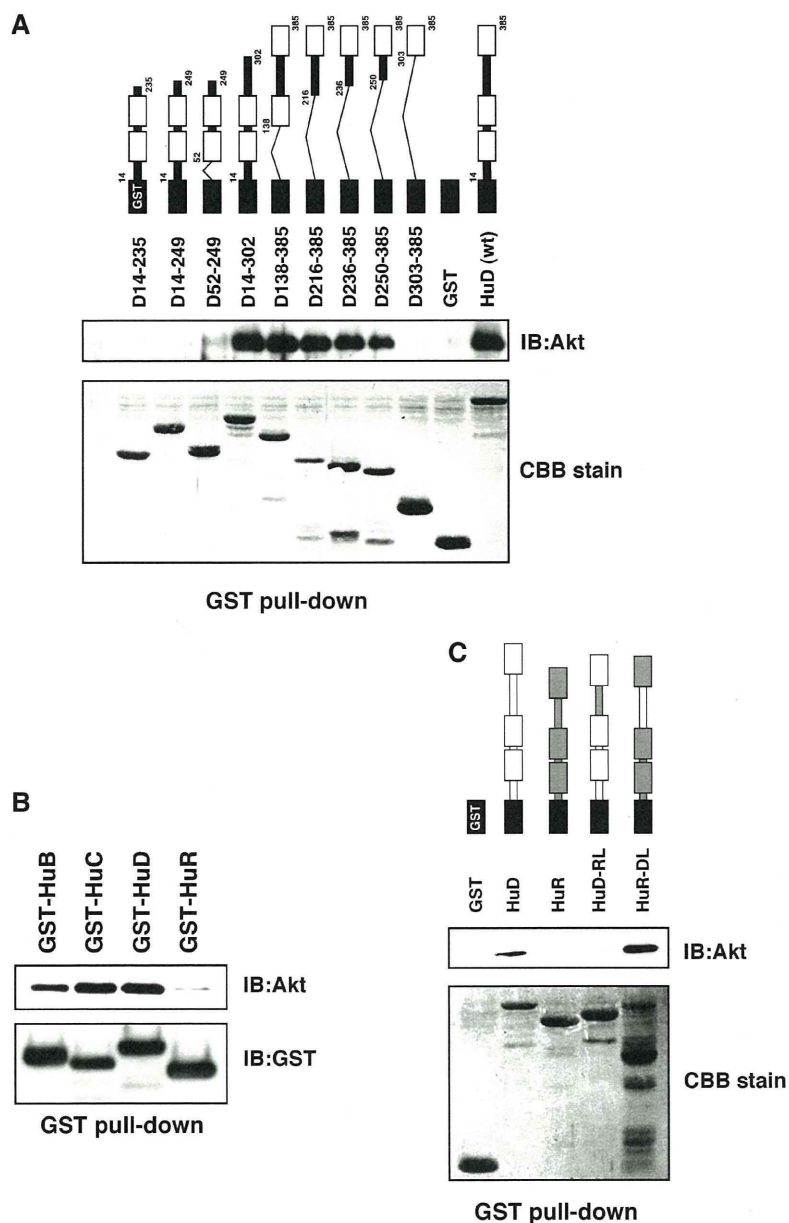
#### Linker region of HuD is required for the interaction of HuD with Akt1

Next, we delineated the HuD sequence elements that are important for its interaction with Akt1 (Figure 3A). Using GST-HuD deletion mutants, we find that the linker region between amino acids 250 and 302 is required for the

optimal interaction of HuD with Akt1 (Figure 3A). Interestingly, the overall amino acid sequence of Hu proteins is well conserved (4). However, the linker region of Hu proteins differs between the neuronal Hu proteins HuB, HuC and HuD and the ubiquitously expressed HuR (Supplementary Figure S2). HuR lacks a short sequence within the linker region, which is found in all neuronal Hu proteins (Supplementary Figure S2). This has prompted us to determine whether Akt1 interacts specifically with the neuronal Hu proteins but not with HuR. Indeed, Akt1 interacts with HuB, HuC and HuD but not with HuR (Figure 3B). To confirm the importance of the linker region in Akt1 binding, we generated chimeric mutants of HuD and HuR, which contain the reciprocal linker between RBD2 and RBD3, respectively (Figure 3C). As predicted, HuR carrying the HuD linker region gains the ability to bind Akt1 whereas HuD, containing the HuR linker regions loses its ability to interact with Akt1 (Figure 3C).

#### Role of Akt1 in HuD-induced neurite outgrowth in PC12 cells

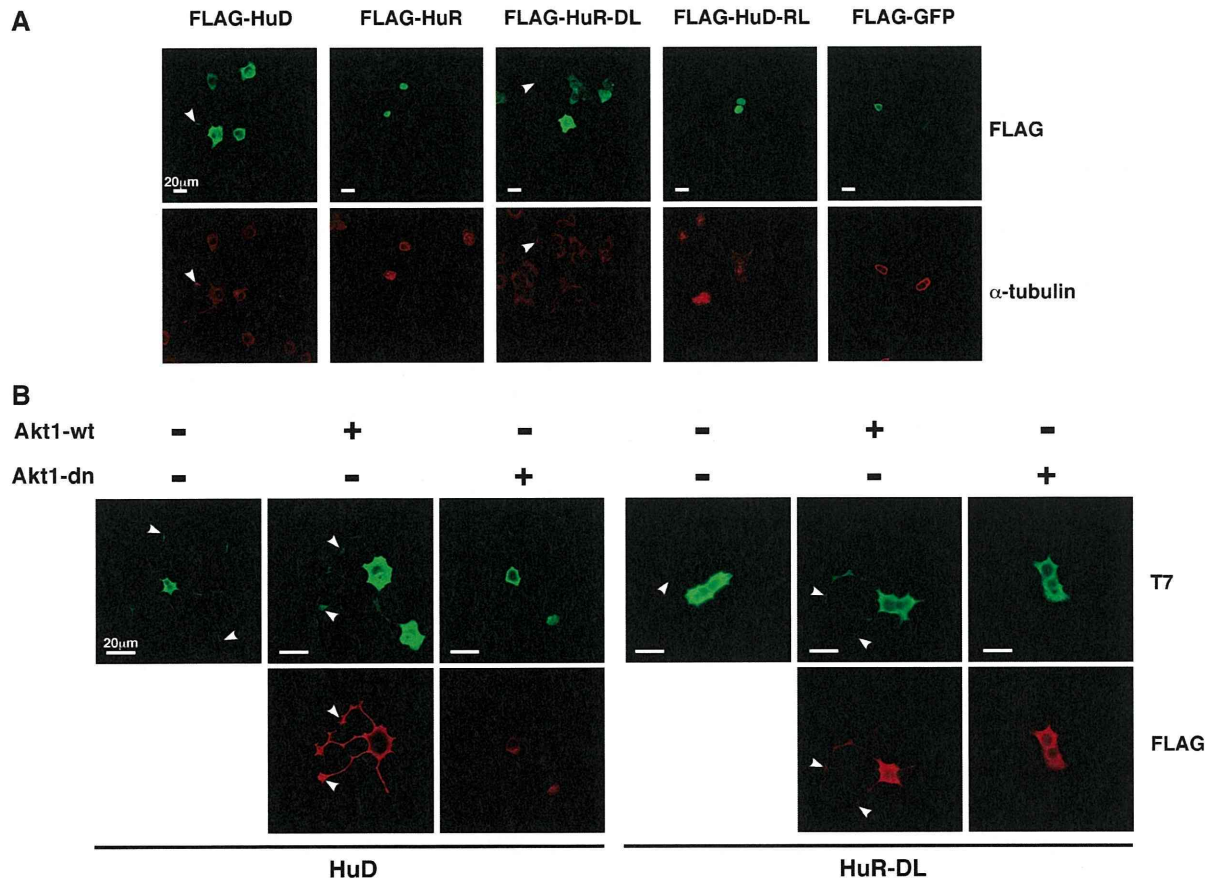
We next wished to test whether the HuD-Akt1 interaction is functionally important. To address this question, we



**Figure 3.** The linker region between RBD2 and RBD3 is required for HuD to interact with Akt1. (A) Akt1 was incubated with the indicated GST-HuD proteins. GST pull-downs were examined for copurification of Akt1 by IB (upper panel) and for pull-down efficiency by CBB staining (lower panel). The white rectangles represent the RBDs of HuD. (B) Akt1 specifically interacts with neuronal Hu proteins. Akt1 was incubated with the indicated proteins. (C) HuD binds Akt1 via the linker region of HuD. Akt1 was incubated with the indicated proteins.

used an established cell-based model system for studying neuronal differentiation, which is based on PC12 cells (13,14). If the HuD–Akt1 interaction is contributing significantly to the function of HuD, then our data predict that the chimeric HuR mutant should gain and the chimeric HuD mutant should lose its ability to induce neurite outgrowth. To test this prediction, we assayed the ability of the different HuD and HuR mutants described above to induce outgrowth in PC12 cells. As shown in Figure 4A and in concert with the data described above,

wild-type HuD and the chimeric mutant HuR but not wild-type HuR or the chimeric HuD mutant transfected into PC12 cells can induce neurite outgrowth. These results suggest that binding of HuD to Akt1 is critical for the neurite-inducing activity of HuD. We note that wild-type HuD and the chimeric mutant HuR-DL that promotes neurite outgrowth are cytoplasmic, while those proteins that are not able to do so (wild-type HuR and the chimeric HuD mutant) are nuclear. To confirm that the Akt1 interaction with HuD and not just the cytoplasmic



**Figure 4.** Role of the HuD–Akt1 interaction in neurite outgrowth. (A) Role of the linker region of HuD to induce neurite outgrowth. Shown is the confocal analysis of PC12 cells that have been transfected with constructs coding for the indicated proteins. Cells were costained with anti-FLAG (green) and with anti- $\alpha$ -tubulin antibody (red). Arrow heads point to induced neurites. Scale bar, 20  $\mu$ m. The same results were obtained in at least three independent experiments. (B) Role of Akt1 in neuronal differentiation in PC12 cells induced by HuD and HuR-DL. Confocal analysis of PC12 cells that were transfected with constructs coding for the indicated proteins. Arrow heads point to induced neurites. Scale bar, 20  $\mu$ m. The same results were obtained in at least three independent experiments.

location is the relevant component, we have further mapped linker region of HuD to identify the critical interaction domain. We find that the HuD mutant 277-385 containing a truncated linker region (277-302) and RBD3 is still capable of (i) interacting with Akt1 (Figure 5B) and (ii) inducing neurite outgrowth in PC12 cells (Figure 5C). Strikingly, we find that the truncated HuR mutant 219-327 containing an almost identical linker sequence (see scheme in Figure 5A for comparison) and RBD3 cannot interact with Akt1 (Figure 5B) and cannot induce outgrowth although it localizes to the cytoplasm (Figure 5C). Thus, we are confident to conclude that the interaction of HuD with Akt1 is the relevant component and not just the cytoplasmic localization.

To further test whether the induction of outgrowth correlates with Akt1 binding and function, we used a dominant negative mutant of Akt1 that is defective in ATP binding and has a phosphorylation site defect within the kinase domain (see ‘Materials and Methods’ section for further details). Binding of this inactive mutant to HuD is strongly reduced (Supplementary Figure S3). The results shown in Figure 4B further

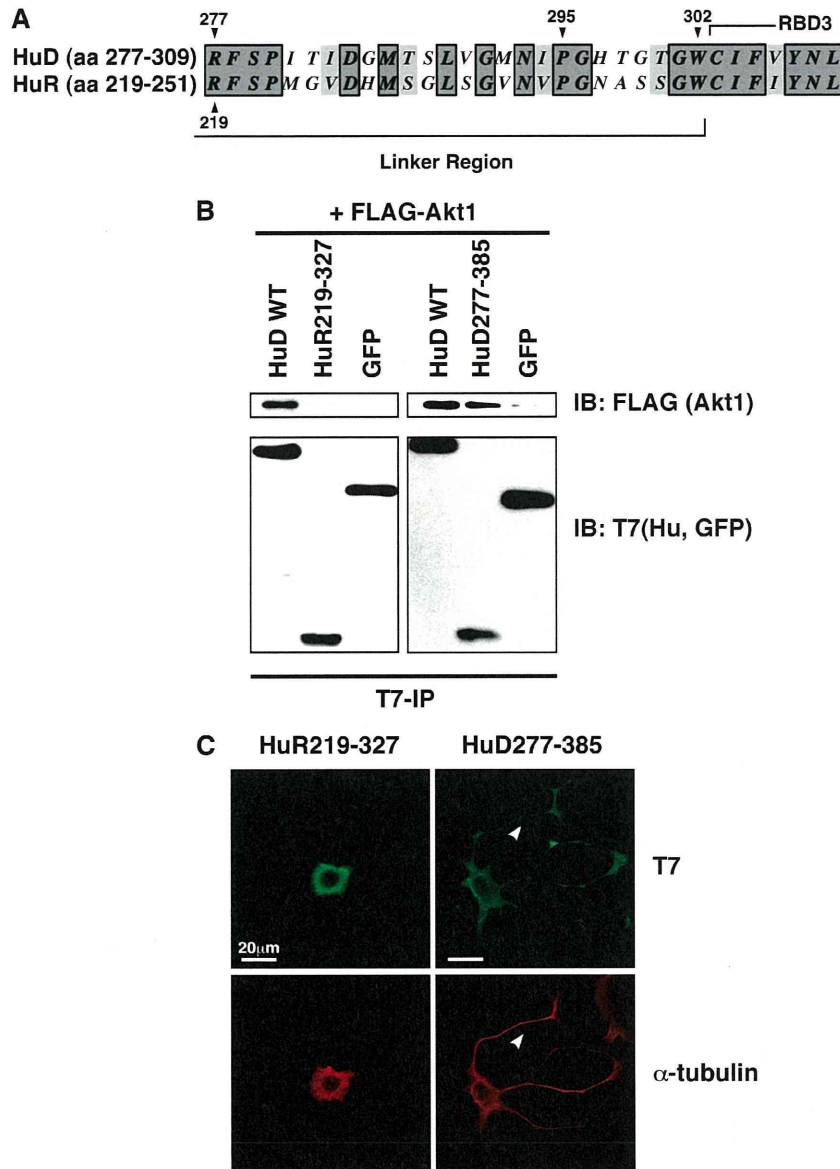
confirm the importance of Akt1 in HuD-induced neurite outgrowth as overexpression of dominant-negative Akt1 but not wild-type Akt1 inhibits neurite outgrowth induced by both HuD and the chimeric mutant HuR-DL.

#### RNA-bound HuD interacts with active Akt1

As all of the biological functions of HuD are believed to be a result of its ability to bind to RNA, we tested whether RNA-bound HuD can associate with Akt1. To address this question, we performed pull-down assays using poly(U) sepharose beads. Indeed, active but not inactive Akt1 associates with RNA-bound HuD (Figure 6A). This binding is specific, because it is not observed with GST or GST-HuR (Figure 6A). Importantly, active Akt1 associated with RNA-bound HuD retains its kinase activity (Figure 6B).

#### HuD itself is not the substrate for Akt1

We finally asked whether HuD is itself substrate for Akt1 phosphorylation or whether HuD recruits the kinase to a different substrate. To address this question, we have

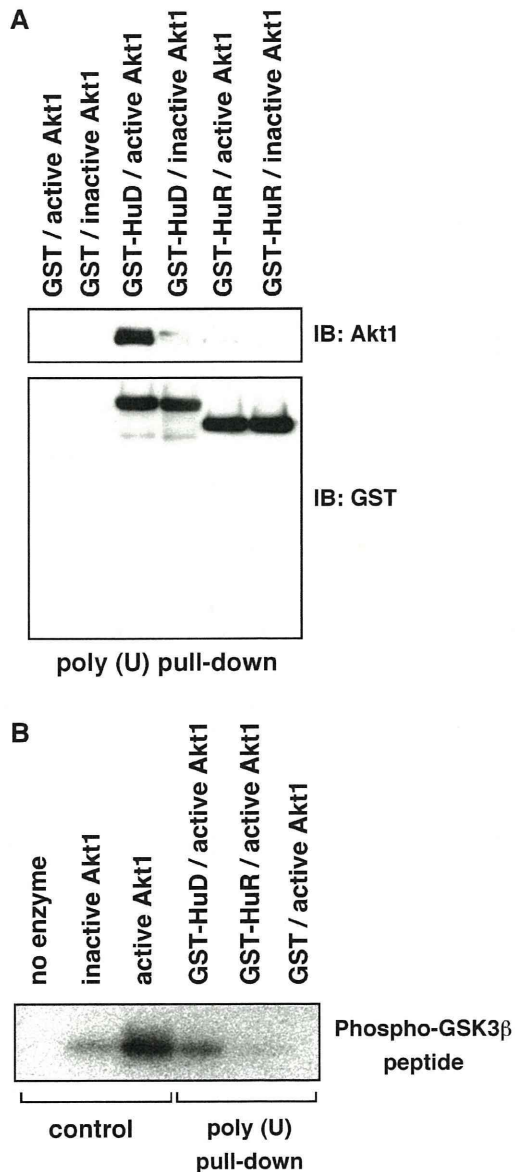


**Figure 5.** HuD 277-385 but not HuR 219-327 binds to Akt1 and induces neurite outgrowth in PC12 cells. (A) Amino acid alignment of the truncated linker and the start of the RBD3 sequence of the mutant HuD 277-385 (amino acids 277-309 is shown) and the mutant HuR 219-327 (amino acids 219-251 is shown). The amino acid sequence of HuD is compared with that of HuR. Identical residues are shown in gray. The linker region is indicated. (B) Specific coimmunoprecipitation of Akt1 with HuD 277-385 but not HuR 219-327. HeLa cells were transfected with the indicated constructs. Immunoprecipitated was performed with anti-T7 antibody. Coimmunoprecipitation was monitored by IB. GFP is a negative control. (C) HuD 277-385 but not HuR 219-327 induces neurite outgrowth in PC12 cells. Shown is the confocal analysis of PC12 cells that has been transfected with constructs coding for the indicated proteins. Cells were costained with anti-T7 (green) and with anti- $\alpha$ -tubulin antibody (red). Arrow heads point to induced neurites. Scale bar, 20  $\mu$ m. The same results were obtained in at least three independent experiments.

performed an *in vitro* kinase reaction using GST-HuD and active Akt1 (see 'Materials and Methods' section for further details). The data shown in Figure 7 reveal that HuD is not phosphorylated by Akt1, whereas the positive control FOXO4 is. On the basis of these results, we conclude that HuD itself is not the target of Akt1. Taken together, with the results described above, our data suggest the model whereby RNA-bound HuD recruits Akt1 to activate neurite outgrowth.

## DISCUSSION

Neuronal Hu proteins are RBPs that are critical for neuronal development (16). However, the underlying molecular mechanisms how neuronal Hu proteins exert their function are poorly understood. We have recently shown that HuD, a family member of neuronal proteins, stimulates cap- and poly(A)-dependent translation and found that the eIF4A and poly(A) binding



**Figure 6.** RNA-bound HuD interacts with active Akt1 and retains its kinase activity. (A) RNA-bound HuD interacts with active Akt1. Active or inactive Akt1 was incubated with GST, GST-HuD or GST-HuR, pulled down with poly(U)-sepharose beads and analyzed by IB and for pull-down efficiency (lower panel) by IB. (B) Akt1 retains its protein kinase activity in the Akt1-HuD-RNA complex. Active Akt1 was incubated with GST-HuD, GST-HuR or GST and then pulled down with poly(U)-sepharose beads. Protein kinase assays were performed using a GSK-3 $\beta$  peptide, which is a specific substrate for Akt1. Controls are standard assays with 'no enzyme', 'inactive Akt1' or 'active Akt1'. In the pull-down lanes approximately 10% of Akt1 has been used for the kinase assay as estimated by western blot analysis (data not shown).

domains which are key in this process also contribute to its neurite-inducing activity (4). Here, we further investigated the underlying molecular interactions and show that HuD directly and specifically interacts with active Akt1. This interaction is RNA independent,

mediated by the linker region between RBD2 and RBD3 and required for its neurite inducing activity (Figures 2–5). We also tested for the interaction of HuD with downstream targets of Akt1 such as FLAG-mTOR or FLAG-S6K1 in HuD immunoprecipitates. However, we could not detect these downstream targets in the eluates (data not shown). Therefore, it appears that HuD interacts with Akt1 specifically and this interaction does not extend to other closely related AGC family kinases.

The strategy of an RBP to interact with a signaling pathway component has some similarity to that of the splicing factor 2 (SF2)/ASF which recruits mTOR or protein phosphatase 2A to activate translation of a subset of mRNAs (17). Interestingly, the finding that the eIF4A and poly(A) domains of HuD are not only critical for HuD's enhancer function in translation but also contribute to its neurite-inducing activity, strongly suggests that stimulation of translation is a prerequisite for the neurite inducing activity of HuD. This raises the possibility that the HuD-Akt1 interaction might underlie a function in regulating translation. eIF4A, which interacts with HuD (4), may help to recruit the HuD/Akt1 dimer into messenger ribonucleoprotein (mRNP) complexes to phosphorylate target molecules to stimulate translation.

What are the targets of Akt1 in such HuD-containing mRNP complexes? We found that HuD itself is not a direct target of Akt1 (Figure 7). A prime candidate target is eIF4B, which can be phosphorylated by Akt1 (Supplementary Figure S4) (18–20) and can stimulate the helicase activity of eIF4A (21,22) and as a consequence translation. Thus, eIF4A activity might indirectly be enhanced by the action of Akt1 kinase via phosphorylation of an activator of eIF4A (e.g. eIF4B) rather than directly, as eIF4A is known not to be phosphorylated. Future experiments will aim to directly address the question of whether tethered HuD enhances cap-dependent translation via regulating the phosphorylation status of eIF4B by recruiting Akt1 into translation mRNP complexes.

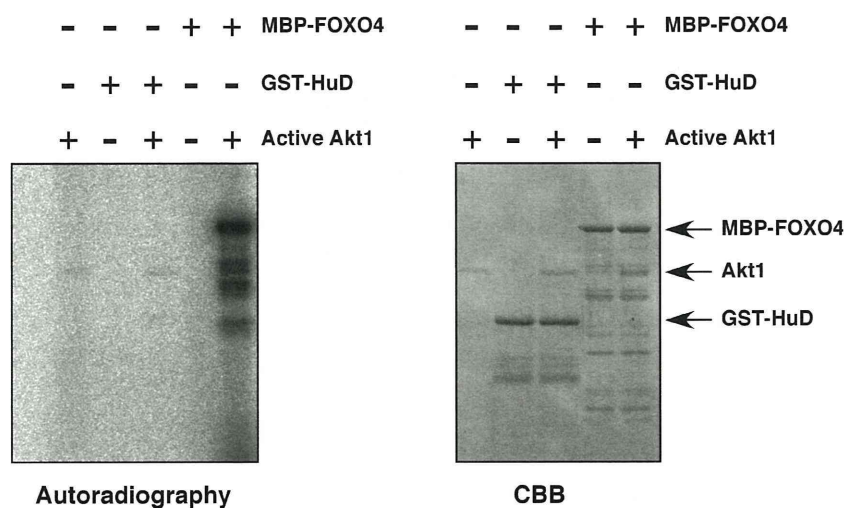
Taken together, our study uncovers a functional interaction between a key RBP involved in neuronal development and a key signaling molecule, which is a master modulator of translation. To our knowledge, this is the first demonstration of a signaling pathway kinase that is specifically recruited by an RBP to trigger neurite outgrowth. In addition, these data might help to explain how HuD enhances translation of mRNAs that encode proteins involved in neuronal development.

#### SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Figures S1–S4.

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**Figure 7.** HuD is not a substrate of Akt1. *In vitro* kinase reaction using recombinant GST-HuD or MBP-FOXO4 and active Akt1 (see 'Material and Methods' section for further details). Phosphorylation levels are shown in the left panel, CBB stain is shown in the right panel. The positions of MBP-FOXO4, Akt1 and GST-HuD are indicated by arrows.

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