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RESEARCH ARTICLE

PIN1 is a novel interaction partner and a negative upstream regulator of the transcription factor NFIB

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NFIB is a transcription factor of the Nuclear Factor One (NFI) family that is essential for embryonic development. Post-translational control of NFIB or its upstream regulators have not been well characterized. Here, we show that PIN1 binds NFIB in a phosphorylation-dependent manner, via its WW domain. PIN1 interacts with the well-conserved N-terminal domains of all NFIs. Moreover, PIN1 attenuates the transcriptional activity of NFIB; this attenuation requires substrate binding by PIN1 but not its isomerase activity. Paradoxically, we found stabilization of NFIB by PIN1. We propose that PIN1 represses NFIB function not by regulating its abundance but by inducing a conformational change. These results identify NFIB as a novel PIN1 target and posit a role for PIN1 in post-translational regulation of NFIB and other NFIs.

Keywords: NFIB; PIN1; post-translational regulation; protein–protein interactions; transcription factor

Embryonic development entails complex cellular events regulated by a variety of signaling pathways and coordinated actions of numerous transcription factors. One of these transcription factors is Nuclear Factor One B (NFIB) of the NFI family which includes three other members: NFIA, NFIC, and NFIX. These proteins have been initially identified because of their role in adenovirus replication [1,2], and indeed, recently NFIB was found to be associated with the pre-replication complex [3] and facilitate chromatin accessibility *in vitro* and *in vivo* [3–6]. Previous work has shown that all NFIs are involved in the regulation of stem cell maintenance and differentiation, dysregulation of which can result in developmental defects as well as cancer [7,8]. For example, we have found that NFIB is required for cell survival in a neuroblastoma cell line [9] while others have shown NFIA or NFIB are necessary for *in vivo* growth of glioblastoma subtypes and maintenance of core transcriptional programs [10].

The typical NFI protein structure comprises a highly conserved N-terminal DNA binding and dimerization (DBD) domain and a less conserved C-terminal transcription activation/repression (TAR) domain [11,12]. In mice, the similarity of C-terminal domains ranges from 40% to 60%, while the overall amino acid sequence homology between NFIs varies between 61% and 73% [12]. Likewise, human NFIs are from 76% (NFIA-NFIC) to 81% (NFIA-NFIB and NFIB-NFIX) identical (Uniprot IDs: Q12857 NFIA, O00712 NFIB, P08651 NFIC, Q14938 NFIX). NFIs bind to the palindromic consensus sequence (TTGGC(N5) GCCAA) as homodimers or heterodimers with similar

Abbreviations

CHX, cycloheximide; CIP, calf intestinal alkaline phosphatase; CNS, central nervous system; DBD, DNA binding and dimerization; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; GFAP, glial fibrillary acidic protein; GST, glutathione *S*-transferase; IgG, immunoglobulin G; IP, immunoprecipitation; KO, knockout; MOI, multiplicity of infection; NFI, nuclear factor one; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PEI, polyethyleneimine; PPIase, peptidyl prolyl isomerase; SDS, sodium dodecyl sulfate; TAR, transcription activation/repression; TBS, tris-buffered saline; TF, transcription factor; WB, western blotting; Y2H, yeast two-hybrid.

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affinity [13]. With their highly conserved DBD domain, NFIs could potentially bind and activate the same target genes with similar potency as supported by *in vitro* evidence [14,15] while C-terminal domains could differentiate NFIs, allowing for more substantial differences in target regulation [16,17].

Mice knockout (KO) studies together with reports on human NFI haploinsufficiency phenotypes support overlapping but distinct functions for NFI proteins during development [8,18,19]. In the cerebral cortex, NFIA, NFIB, and NFIX are expressed in radial glial cells [20–22] and disruption of each gene gives rise to delayed differentiation and enlarged ventricles as well as an abnormal corpus callosum [20–28]. The severity of these cortical phenotypes in double heterozygous ($Nfia^{+/-}$; $Nfib^{+/-}$) and homozygous ($Nfia^{-/-}$ and $Nfib^{-/-}$) KOs are similar; while double homozygous ($Nfia^{-/-}$;$Nfib^{-/-}$) KOs exhibit exacerbated defects, supporting an additive model for NFI function [29]. The similarity between *Nfi* null cortical phenotypes is reproduced in humans as NFIA, NFIB, or NFIX haploinsufficiency all lead to macrocephaly, agenesis of the corpus callosum, and other neurodevelopmental deficits. Moreover, outside of the central nervous system (CNS), *Nfi* null mice display distinct phenotypes: *Nfia* KO mice develop urinary tract and kidney defects [30], the absence of NFIB leads to respiratory failure resulting from delayed lung maturation [27] whereas *Nfix* KO mice show muscle and skeletal anomalies [31,32]. Overall, these studies underline the non-redundant roles of NFI genes in embryonic development.

Currently, whether each NFI acts distinctly or if their overall effect is additive has not been resolved. Indeed, while there is significant overlap between the *in vivo* binding sites and target genes of individual NFIs [8,29,33], there appears to be a distinct set of genes specifically regulated by each member. These differences could be due to their divergent C termini conferring each NFI a unique domain that can interact with a different repertoire of proteins to activate or repress specific target genes [33]. Until recently, only a limited number of NFI interaction partners had been identified [34–38]. However, in a screen designed to investigate interaction networks encompassing transcription factors (TFs), Göös *et al.* [39] found most of the selected 109 bait TFs interacted with NFIs. This study uncovered an extensive set of TF-TF interactions converging on NFIs, linking them to other TF signaling pathways with potential co-regulation of downstream targets. Meanwhile, upstream regulators of NFI proteins are yet to be explored.

PIN1 is a ubiquitously expressed peptidyl-prolyl isomerase (PPIase) that regulates the function, activity, stability, phosphorylation states, or cellular localization of numerous proteins [40–47]. It consists of an N-terminal WW domain, including two highly conserved tryptophan residues, that binds phosphorylated Ser/Thr-Pro residues and a C-terminal domain that catalyzes cis/trans isomerization of target proteins, thereby changing the conformation of proteins [48,49]. As PIN1 can catalyze post-phosphorylation conformational changes in major molecular players involved in cell cycle and growth, it acts as an important regulator of oncogenic signaling pathways, and in turn, tumor development and cancer [42,50]. Interestingly, PIN1 levels are increased in multiple cancers including glioblastoma [51–54]. Consequently, upregulation of PIN1 may enhance the activity or abundance of oncogenes while decreasing that of tumor suppressors [41,55,56], implicating PIN1 in tumor progression.

In order to identify novel interaction partners which may regulate NFIs in the developing CNS, we have previously performed a yeast two-hybrid (Y2H) screen using human fetal brain cDNA library. Using NFIB as bait, PIN1 was determined to be a strong candidate interaction partner. Both NFIB and PIN1 are expressed by neural stem cells within ventricular zones [22,40,44], are required for proper CNS development [20,57], and are found in the nucleus [33,40], and misregulation of both NFIB and PIN1 is implicated in various cancers [7,50]. Based on this functional overlap, we set out to investigate NFIB-PIN1 interactions and found that PIN1 binds NFIB as well as the other NFIs when overexpressed in HEK293T cells, and the NFIB-PIN1 interaction occurs in a phosphorylation-dependent manner. PIN1 recognizes the N-terminal DBD domain of NFI family members via its WW domain. We surmise that NFIB binding to PIN1 leads to a conformational change which then leads to a decrease in transcriptional activity. We have also found evidence that PIN1 increases NFIB's stability. Taken together, these results point to a novel interaction between PIN1 and NFIB, and a role for PIN1 as an upstream regulator of NFIB during development of CNS and other organ systems as well as in cancers afflicting these same organ systems.

Materials and methods

Plasmids

pCHNFI-A4, pCHNFI-B2, pCHNFI-C2, and pCHNFI-X2 constructs were gifts from Richard Gronostajski (Addgene plasmids #31404, 31405, 31403, 31302) [58]. The construct encoding the hemagglutinin (HA) tagged NFIB N-terminal domain was generated as described previously [9]. cDNAs encoding NFIB C-terminal domain and the N-terminal

domains of NFIA, NFIC, and NFIX were inserted in frame with the HA-tag of pCHNFI-B2 between the restriction sites *SfiI* and *BglII*. GST-PIN1 was a gift from Michael Yaffe (Addgene plasmid #19027) [59]. Flag-tagged PIN1 encoding cDNA was inserted between *XhoI* and *BamHI* restriction sites of pcDNA 3.1. Lentiviral packaging plasmids were gifts from Didier Trono (Addgene plasmids #12259, #12251, #12253) [60]. Human PIN1 knockdown targeting sequence, shPIN1: 5′-CCACCGTCACACAGTATTTAT-3′ was retrieved from Broad Institute RNAi consortium (shRNA TRCN0000001033). A non-targeting sequence was used also as control, shControl: 5′-CAACAAGATGAAGAGCACCA A-3′ [61]. Both shRNA sequences were cloned into the pLKO.1-TRC-puro vector, which was restricted with *AgeI* and *EcoRI* as described previously [9]. All cloning reactions utilized homologous recombination using the In-Fusion HD cloning kit (638910; Takara, Mountain View, CA, USA) according to the manufacturer's instructions. All constructs were verified by Sanger sequencing (Macrogen Biotech, Amsterdam, The Netherlands).

Mutagenesis

Point mutations in FLAG-PIN1 and HA-NFIB, and deletion mutations in HA-NFIB were generated using the Q5 Site-Directed Mutagenesis Kit (NEB, Ipswich, MA, USA) according to the manufacturer's instructions.

Cell culture

Human embryonic kidney 293T (HEK293T) (RRID: CVCL_0063) and SH-SY5Y human neuroblastoma cells (RRID: CVCL_0019) were originally purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cell lines have undergone authentication within the past 3 years using short tandem repeat (STR) analysis and were consistently maintained free from mycoplasma contamination throughout all experiments. HEK293T cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco), 2 mM glutamine (Gibco), 100 $U \cdot mL^{-1}$ penicillin (Gibco), and 100 $\mu g \cdot mL^{-1}$ streptomycin (Gibco), at 37 °C in a humidified atmosphere of 5% CO_2. SH-SY5Y cells were cultured in Dulbecco's modified Eagle's medium: Nutrient's mixture F12 (DMEM/F12) (Gibco) supplemented with 20% fetal bovine serum (Hyclone, Logan, UT, USA), 2 mM Glutamine (Gibco), 100 $U \cdot mL^{-1}$ penicillin, and 100 $\mu g \cdot mL^{-1}$ streptomycin (Gibco).

Lentivirus production and infection

shControl or shPIN1 encoding lentiviruses were packaged in HEK293T cells as described previously [9]. The multiplicity of infection was set as 2 for knockdown experiments. 72 h after the infection, cells were harvested for protein expression analyses. Virus production efficiency was determined by colony formation assay for each batch of virus. Briefly, HeLa cells were transduced with shControl or shPIN1 encoding viruses, and treated with 500 $ng \cdot mL^{-1}$ puromycin for 10 days. Antibiotic-resistant colonies were fixed with ice-cold methanol for 20 min and stained with 0.5% crystal violet in 25% methanol as described previously [9].

Western blotting (WB) and antibody list

Whole-cell extracts were prepared using a lysis buffer including 50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 5 mM EDTA, and 1% NP-40 supplemented with protease inhibitors (4693159001; Roche, Mannheim, Germany). Samples were incubated on ice for 30 min, then collected and centrifuged at 10 000 ***g*** for 10 min at 4 °C. Next, the cell extracts were resolved by SDS/PAGE under reducing conditions and transferred to a nitrocellulose membrane. The membrane was blocked using 3% non-fat dry milk diluted in TBST. The membrane was incubated with one of these primary antibodies: anti-PIN1 (G8, sc-46660, mouse monoclonal; Santa Cruz, Dallas, TX, USA), anti-HA (C29F4, rabbit monoclonal; CST, Danvers, MA, USA), anti-HA (12CA5, mouse monoclonal; Roche), anti-Flag (2368, rabbit polyclonal; CST), anti-GST (ab19256, rabbit polyclonal; Abcam, Cambridge, UK), anti-NFIB (HPA003956, rabbit polyclonal; Sigma, St. Louis, MO, USA), anti-NFIA (ARP32714; Aviva, San Diego, CA, USA), anti-NFIX (ARP32717; Aviva), anti-NFI (H300, sc-5567, rabbit polyclonal; Santa Cruz), anti-GAPDH (6C5, sc-32233, mouse monoclonal; Santa Cruz), or anti-β-Actin (C4, sc47778, mouse monoclonal; Santa Cruz). Next, anti-rabbit IgG-HRP (7074; CST) or anti-mouse IgG-HRP (7076; CST) were used as secondary antibodies. The protein bands were visualized using Westernbright ECL HRP substrate (K-12045-D50; Advansta, San Jose, CA, USA). ChemiDoc MP Imaging System (Bio-Rad, Hercules, CA, USA) was used for image acquisition.

Immunoprecipitation

HEK293T cells were plated in a 100-mm cell culture dish at a density of 2.5×10^6 the day before the transfection, and the next day, co-transfection was performed. Ten microgram plasmid DNA was mixed with 1 $mg \cdot mL^{-1}$ Linear PEI (23966; PolyScience, Warrington, PA, USA) at a 1 : 3 DNA/-PEI ratio. Forty-eight hours after the transfection, cells were lysed with immunoprecipitation (IP) buffer [50 mM Tris-Cl (pH 8.0), 150 mM NaCl, 1% (v/v) NP-40, 5 mM EDTA supplemented with protease inhibitors]. For immunoprecipitation experiments, the cell lysates overexpressing HA- or Flag-tagged proteins were precipitated overnight using α-HA- or α-Flag-conjugated agarose beads (Sigma, A2095, and Sigma, A2220, respectively). To immunoprecipitate endogenous NFIB or PIN1, α-NFI- or α-PIN1-conjugated agarose beads were used (Santa Cruz, D-2, sc-74444; Santa Cruz, G-8, sc-

46660, respectively). The next day, beads were washed three times with IP buffer, and bound proteins were eluted by boiling in 1.5× sample buffer. Eluates were resolved by SDS/PAGE and subjected to WB analysis.

GST pull-down assays

Glutathione *S*-transferase and GST-PIN1 were produced in *Escherichia coli* BL21 strain, and the cells were lysed in BugBuster (70584-3; Novagen, Darmstadt, Germany). GST or GST-PIN1 was bound to Glutathione Sepharose 4B beads (GE-170756-01; GE-Healthcare, Uppsala, Sweden) at room temperature for 1 h. Next, HEK293T cell lysates expressing HA-NFIs or HA-tagged NFIB C-terminal or N-terminal domains were added and incubated further with slow rotation at 4 °C for 2 h. The beads were then washed three times using PBS with 1% Triton-X (PBST). The bound proteins were eluted in 1.5× sample buffer. Eluates were resolved by SDS/PAGE, and subjected to WB analysis.

Luciferase reporter assays

Luciferase reporter plasmid pGfa2-luc3, which carries the *GFAP* promoter (−2163 to +47 with the initiating ATG mutated to TTG), was a gift from Michael Brenner (Addgene plasmid #53129) [62,63]. For luciferase assays, 5×10^4 HEK293T cells were seeded in a 24-well plate. The next day, using Fugene6 transfection reagent (E2691; Promega, Madison, WI, USA), cells were transfected with pGfa2-luc3 as well as expression vectors encoding HA-NFIB and/or FLAG-PIN1 constructs. pRL-TK renilla reporter plasmid (E2241; Promega) was included as normalization control. Forty-eight hours after the transfection, cells were harvested using 1× PLB. The bioluminescence signals of the samples were measured using the Dual-Luciferase Reporter Assay System (E1910; Promega) according to the manufacturer's instructions with the Fluoroskan Ascent FL luminometer microplate reader (Thermo Fisher Scientific, Vantaa, Finland). For each sample, the firefly luciferase activity was normalized to that of renilla luciferase. The samples were measured in triplicates, and the average value was used for further calculations.

Calf intestinal alkaline phosphatase (CIP) treatment

HEK293T cells overexpressing HA-NFIB were lysed with IP buffer, and the cell lysates were precipitated with α-HA-conjugated agarose beads. Precipitates were then incubated with CIP (1 unit·μg^{-1} protein) (M0290; NEB) at 37 °C for 4 h. Next, CIP was removed from the sample by centrifuging the tubes. The samples were washed three times and incubated with GST or GST-PIN1 for 1 h. After the incubation, samples were washed three times with PBST and eluted in 1.5× sample buffer. Then, eluates were resolved by SDS/PAGE, followed by WB analysis.

Protein stability assays

HEK293T cells were transfected with plasmids encoding HA-NFIB together with FLAG-PIN1, or mock vector, and incubated overnight. For the PIN1 knockdown experiments, HEK293T cells were infected with shControl or shPIN1 encoding lentiviruses and transfected with HA-NFIB. The next day, cells were seeded into 24-well plates. The following day, cells were treated with 50 μg·mL^{-1} CHX or DMSO for the indicated hours. After the incubation with CHX or DMSO, cells were lysed in IP buffer. Protein samples were subjected to WB analysis.

Results

Pin1 can interact with all NFI family members

One way by which NFI family of transcription factors can differentially regulate target genes is via interactions with specific co-regulators. To identify novel proteins that bind and regulate the activity of NFIB, we performed a Y2H screen using fetal brain cDNA library. Among 12 colonies that encode a full-length protein, 4 clones corresponded to PIN1 (NM_006221.3). To validate this putative interaction, we first performed GST pull-down assays. HEK293T cells were overexpressed with HA-tagged NFIB and the cell extracts were incubated with bacterially expressed GST or GST-PIN1. We found that HA-NFIB was precipitated with GST-PIN1 but not with GST alone (Fig. 1A). Next, we tested the affinity of GST-PIN1 for the other NFI family members: NFIA, NFIC, and NFIX. GST-PIN1, but not GST alone, consistently pulled down HA-NFIA, HA-NFIC, and HA-NFIX (Fig. 1B,C). To verify these interactions in mammalian cells, we overexpressed FLAG-PIN1 along with HA-NFIA, HA-NFIB, HA-NFIC, or HA-NFIX in HEK293T cells and performed co-immunoprecipitation assays. FLAG-PIN1 co-immunoprecipitated with each NFI, while there was little Flag-PIN1 in anti-HA immune complexes in the absence of NFIB (Fig. 1D). In reciprocal experiments, significant amounts of each NFI family member co-immunoprecipitated with Flag-PIN1, compared to the anti-Flag control (Fig. 1E). These results indicate that PIN1 interacts with each NFI family member in HEK293T cells *in vitro*.

PIN1 interacts with NFI proteins SH-SY5Y cells

As HEK293T cells express relatively low levels of all NFI proteins (Fig. S1), to verify NFIB-PIN1 interactions *in vivo*, we utilized SH-SY5Y neuroblastoma cells which express both proteins [9,64]. We found that

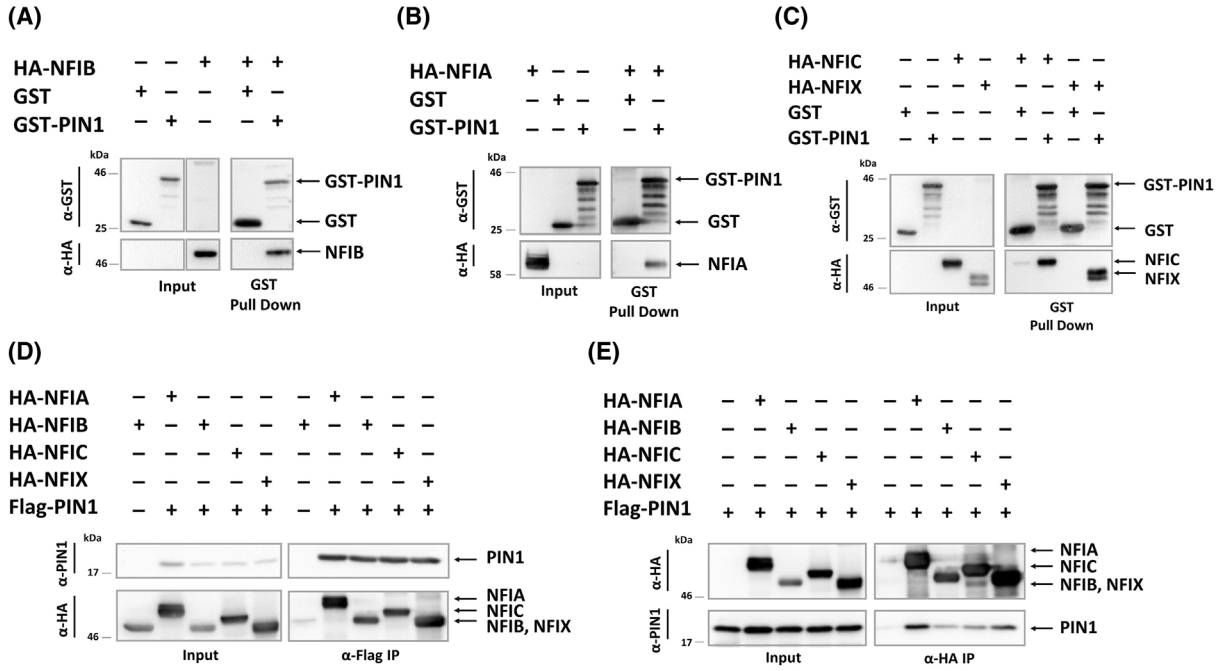

Fig. 1. NFI family members interact with PIN1. (A–C) For GST pull-down analyses, GST or GST-PIN1 was bound to glutathione sepharose and incubated with extracts of HEK293T cells expressing HA-NFIA, HA-NFIB, HA-NFIC, or HA-NFIX. The precipitates were subjected to SDS/PAGE and immunoblotted with α-GST and α-HA antibodies. (D, E) For immunoprecipitation assays HA-NFIs and Flag-PIN1 were co-expressed in HEK293T cells. HA-NFIs and Flag-PIN1 were immunoprecipitated with α-HA- or α-Flag-conjugated agarose beads. The immunoprecipitates were resolved by SDS/PAGE and immunoblotted with α-HA and α-Pin1 antibodies. Input represents 4% of protein lysate used for immunoprecipitation. Arrows indicate the positions of immunoreactive bands of GST, GST-PIN1, HA-NFIs, and Flag-PIN1. Results shown are representative of three independent experiments.

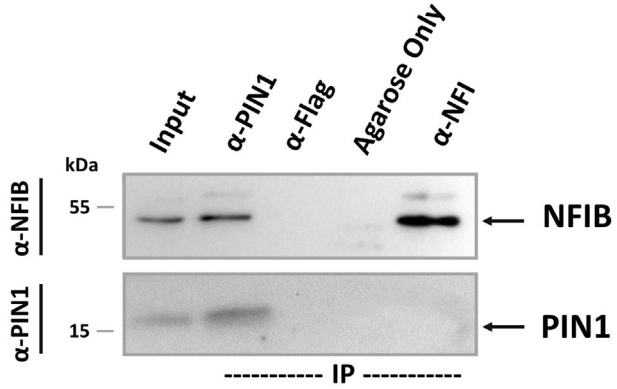

Fig. 2. Endogenous PIN1 interacts with NFI. PIN1 or NFI was precipitated in SH-SY5Y cells, using agarose beads conjugated with α-PIN1 or α-NFI antibodies. Incubation of lysates with α-Flag-conjugated agarose beads and agarose beads only were used as immunoprecipitation controls. Immunoprecipitates were subjected to WB analysis. Arrows point to the positions of immunoreactive bands of endogenous NFIB and PIN1. Data shown are representative of three independent experiments.

endogenously expressed NFI proteins, including NFIB, are co-immunoprecipitated with PIN1 (Fig. 2, Fig. S2). These complexes may involve NFIB homodimers as well as heterodimers of NFIB with other NFI family members, as each member interacts with PIN1 (Figs 1D,E and 2). In reciprocal experiments, we used pan-NFI antibody-conjugated agarose; we found that NFI proteins could not co-immunoprecipitate PIN1, most likely due to steric hindrance by anti-NFI antibodies. Overall, these data show that endogenous PIN1 is associated with endogenous NFI proteins in SH-SY5Y cells.

Pin1 interacts with NFI N-terminal DNA binding and dimerization domains

PIN1 binds overexpressed NFI family members and endogenous NFI protein complexes that include NFIB. Next, in order to identify the NFI protein regions required for PIN1 binding, we searched for PIN1 recognition motifs on NFI proteins. NFIs comprise two domains: the N-terminal DBD domain and the C-terminal TAR domain. NFI N-terminal DBD domains are highly homologous and harbor two conserved Ser/Thr-Pro motifs that can be potentially recognized by PIN1 (Fig. S3). While the C-terminal TAR domains display lower structural homology, they also carry several conserved Ser/Thr-Pro motifs (Fig. S3). Via these motifs, each NFI domain could interact with PIN1. In order to investigate such interactions, we fused an N-terminal HA-tag to individual NFI DBD domains as well as NFIB's TAR domain and co-expressed them along with Flag-PIN1 in HEK293T cells. We found that PIN1 interacts with the N-terminal DBD domain, but not with the C-terminal TAR domain of NFIB (Fig. 3A). GST pull-down analysis further confirmed that the NFIB C-terminal TAR domain has no affinity for PIN1 (Fig. 3B). In reciprocal experiments, N-terminal DBD domains of NFIA, NFIC, and NFIX all interacted with Flag-PIN1 (Fig. 3C,D). These data indicate that NFI N-terminal domains are necessary and sufficient for PIN1 binding.

For subsequent experiments, we focused on NFIB, as PIN1 and NFIB have been both identified as oncogenes in various types of cancer and are likely to co-express and interact in malignant cells which may then have significant consequences for oncogenic signaling pathways in these malignancies [65,66]. Next, we set out to locate the specific NFIB region required for PIN1 binding. To this end, we created NFIB deletion mutants and tested their interaction with PIN1 (Fig. 4A). We found that compared to full-length NFIB, the deletion of residues 1–79 (construct Δ1–79)

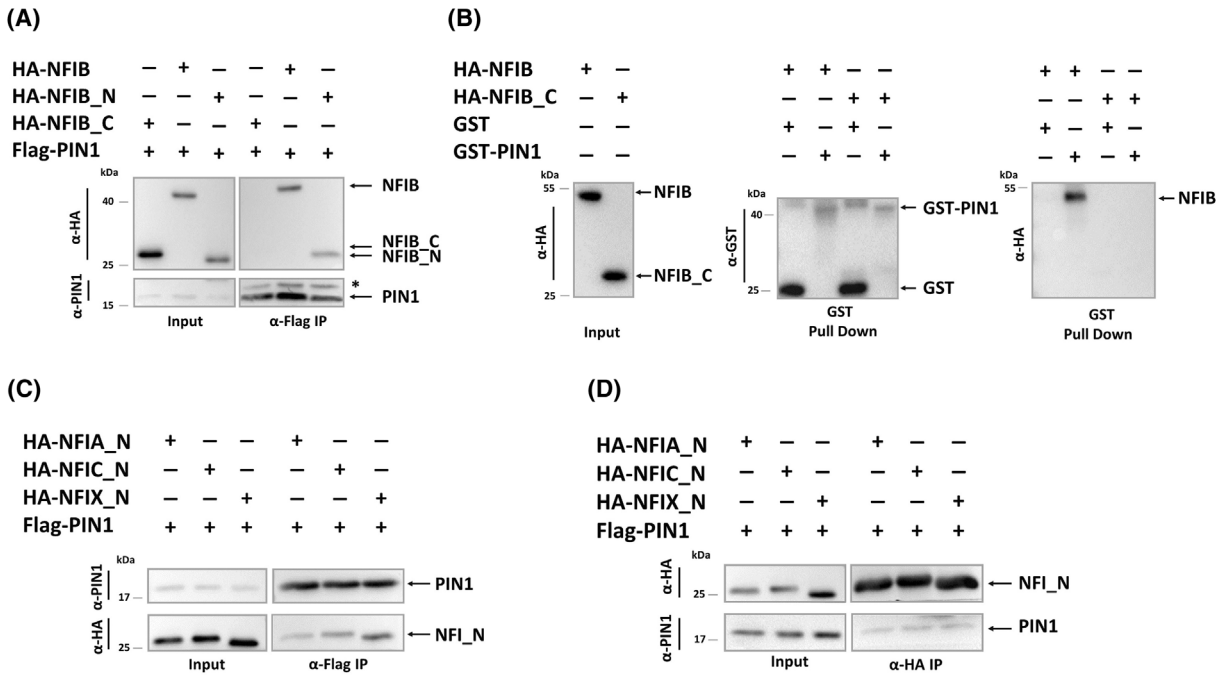

Fig. 3. PIN1 interacts with the N-terminal DBD domain of all NFI family members. (A) HA-NFIB, HA-NFIB N-terminal domain (HA-NFIB_N), or HA-NFIB C-terminal domain (HA-NFIB_C) were co-expressed together with Flag-PIN1 in HEK293T cells. Cell lysates expressing indicated proteins were immunoprecipitated with α-HA- and α-Flag-conjugated agarose beads. Immunoprecipitates were resolved by SDS/PAGE and immunoblotted with α-HA and α-PIN1 antibodies. (B) The lysates of HEK293T cells expressing HA-NFIB or HA-NFIB_C were incubated with GST or GST-PIN1. Precipitates were subjected to SDS/PAGE and immunoblotted with α-GST and α-HA antibodies. The left panel in (B) shows a longer exposure. (C, D) α-HA- and α-Flag-conjugated agarose beads were used to immunoprecipitate the lysates of HEK293T cells expressing HA-NFIA N-terminal domain (HA-NFIA_N), HA-NFIC N-terminal domain (HA-NFIC_N), or HA-NFIX N-terminal domain (HA-NFIX_N) together with Flag-PIN1. Immunoprecipitates were subjected to SDS/PAGE and immunoblotted with α-HA and α-PIN1 antibodies. Arrows indicate the positions of immunoreactive bands of HA-NFIB, HA-NFI domains, Flag-PIN1, GST, and GST-PIN1. Asterisk indicates IgG light chain bands recognized by secondary anti-mouse antibodies (A). Data shown in A and B are representative of three independent experiments.

has no obvious effect on PIN1 binding (Fig. 4B, lanes 3, 7). Moreover, NFIB mutant lacking residues 80–166 (construct Δ80–166), also appears to interact with similar affinity (Fig. 4B, lanes 4, 8). In contrast, the removal of residues 167–208 from the full-length protein (construct Δ167–208), markedly reduces PIN1 binding (Fig. 4C, compare lanes 6–8). Furthermore, while the NFIB C-terminal TAR domain (construct Δ1–209) cannot bind PIN1 at all (Fig. 3A,B), mere addition of residues 167–208 creates a mutant protein that can interact with PIN1 (Fig. 4D). Thus, while we cannot definitively overrule involvement of residues 80–166 in the interaction, the region between residues 167–208 appears to render NFIB a substrate for PIN1 (Fig. 4C,D). Taken together, these data suggest that PIN1 binding site(s) may reside in region 167–208 on NFIB.

PIN1 interacts with NFIB via its WW domain in a phosphorylation-dependent manner

PIN1 comprises a substrate-binding N-terminal WW domain (1–39 aas) and a C-terminal catalytically active PPIase domain (50–163 aas). These two domains are connected by a flexible linker which allows them to communicate with each other [67]. In order to map PIN1 functional domains involved in NFI interactions, we made use of established PIN1 mutants carrying point substitutions in the substrate binding domain (S16D, W34A) and in the catalytic domain (K63A, or C113A) that render these domains non-functional [68]. Expectedly, PIN1 mutants with W34A or S16D substitutions failed to co-immunoprecipitate with NFIB (Fig. 5A,B), whereas PIN1-K63A or PIN1-C113A mutants did not change in their affinity for NFIB (Fig. 5C). Several studies have shown that PIN1 can either bind to its substrates in a phosphorylation-dependent [41,56,69] or independent manner [70–72]. Therefore, to determine whether the PIN1-NFIB interaction is phosphorylation-dependent, we subjected immunoprecipitated HA-NFIB to dephosphorylation by CIP. GST or GST-PIN1 were then incubated with CIP-treated or CIP-untreated NFIB. We found that CIP-treated NFIB precipitates a lesser amount of GST-PIN1 compared to CIP-untreated NFIB, suggesting that NFIB-PIN1 binding is at least partially mediated by phosphorylation (Fig. 5D). As PIN1 generally recognizes the phosphorylated Ser/Thr-Pro motif on its target proteins, we made mutations in NFIB, converting all eight conserved Ser/Thr-Pro residues to Ala-Pro (Figs S3 and S4A), and assessed the resulting NFIB mutants' interaction with PIN1. Surprisingly, mutation of all conserved eight Ser/Thr-Pro motifs did not abolish PIN1-NFIB interaction, suggesting that

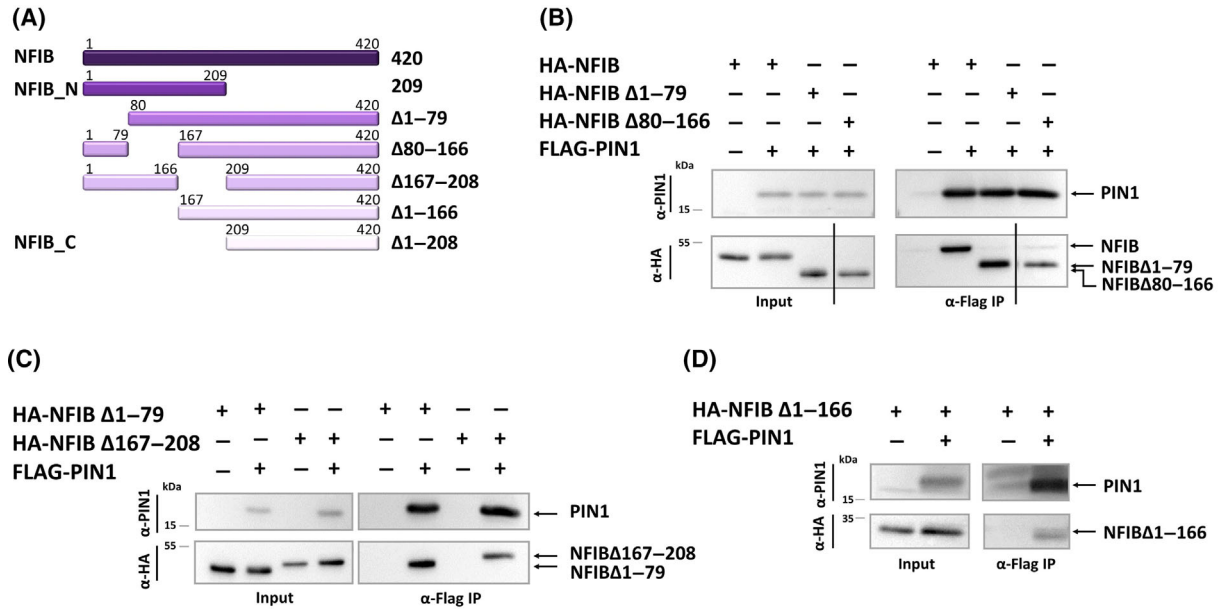

Fig. 4. Two potential binding sites for PIN1 reside in the N-terminal domain of NFIB. (A) Schematic representation of full-length NFIB and NFIB deletion constructs. (B–D) α-Flag-conjugated agarose beads were used to precipitate the lysates of HEK293T cells expressing Flag-PIN1 together with HA-NFIB Δ1–166, HA-NFIB Δ80–166, HA-NFIB Δ1–79, or HA-NFIB Δ167–208. Immunoprecipitates were resolved by SDS/PAGE and immunoblotted with α-HA and α-PIN1 antibodies. Lanes 4 and 8 in the lower panel show a longer exposure (B). Results shown are representative of three independent experiments.

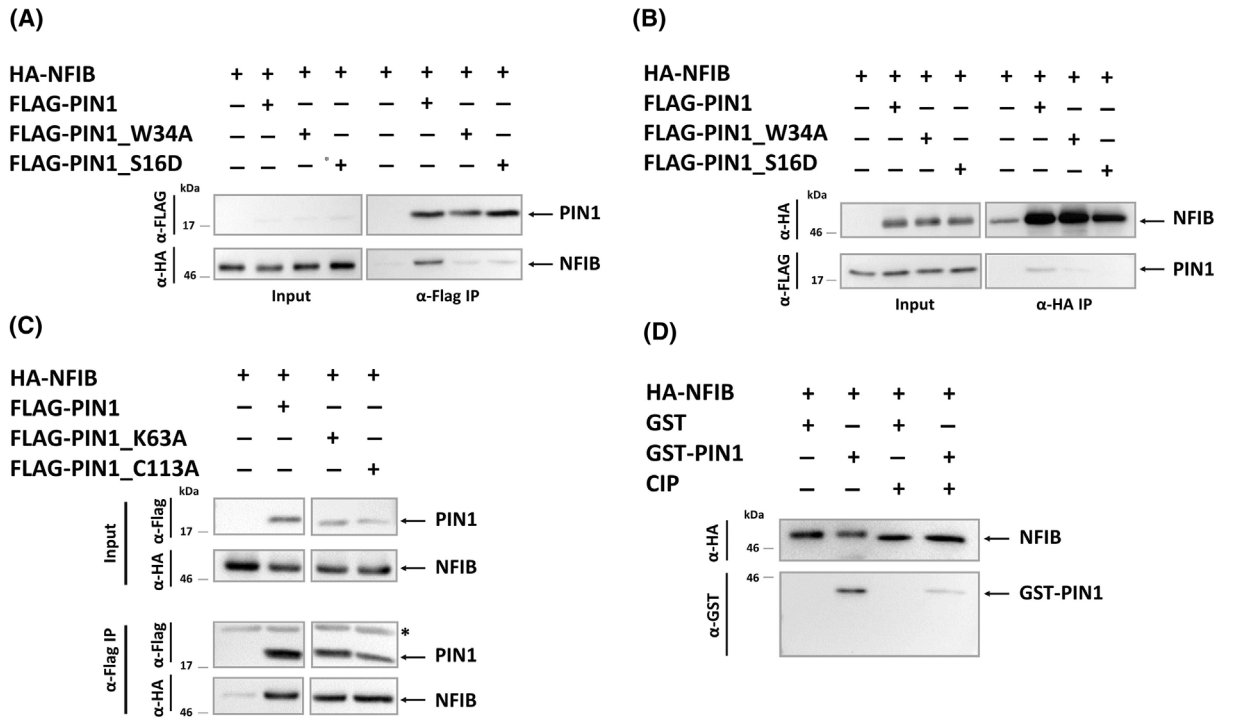

Fig. 5. PIN1 interacts with NFIB via its WW domain in a phosphorylation-dependent manner. (A–C) HEK293T cells were co-transfected with HA-NFIB together with wild-type Flag-PIN1 or Flag-PIN1 mutants. HA-NFIB or Flag-PIN1 was immunoprecipitated with α-HA- and α-Flag-conjugated agarose beads, respectively. Immunoprecipitates were then resolved by SDS/PAGE and immunoblotted with α-Flag and α-HA. (D) HA-NFIB was overexpressed in HEK293T cells and immunoprecipitated with α-HA agarose beads and treated with CIP. Then, CIP-treated or untreated samples were incubated with GST or GST-PIN1 and analyzed by SDS/PAGE. Asterisk indicates IgG light chain bands recognized by secondary anti-mouse antibodies (C). The bottom right panel in (B) shows a longer exposure. Results shown are representative of three independent experiments.

non-canonical motifs in NFIB are responsible for or may also contribute to PIN1 binding (Fig. S4B–D). Taken together, the results show that PIN1 recognizes NFIB via its WW domain, NFIB-PIN1 interaction occurs in a phosphorylation-dependent manner and may require non-canonical motifs for PIN1 recognition.

PIN1 represses NFIB-driven *GFAP* activation

To test whether PIN1 regulates the transcriptional activity of NFIB, we chose the well-characterized promoter of *GFAP*, that is activated by NFIs [73]. We measured *GFAP* promoter-driven luciferase activity in the presence or absence of Flag-PIN1. As expected, HA-NFIB alone induced the *GFAP* promoter (Fig. 6A). Interestingly, overexpressed Flag-PIN1 repressed HA-NFIB-driven *GFAP* activation, whereas substrate-binding-deficient PIN1-W34A did not do so (Fig. 6A). Additionally, catalytic inactive mutants PIN1-K63A and PIN-C113A, which interact with NFIB, functionally represses NFIB as potently as wild-type PIN1 (Fig. 6A), suggesting that PIN1 may modulate transcriptional activation by NFIB. PIN1 may regulate transcription factors by altering their abundance [74] and may act similarly on NFI proteins. To address this possibility, we varied the amount of overexpressed NFIB in the presence of PIN1 and compared the GFAP activation levels. However, increasing exogenous NFIB levels (Fig. 6B, bottom panel, lanes 1, 4) could not reverse the PIN1-mediated repression of *GFAP* promoter activation (Fig. 6B), suggesting that PIN1's attenuation of NFIB's transcriptional activity is not solely dependent on NFIB protein levels and instead may arise due to conformational changes in NFIB. Alternatively, PIN1 may be indirectly regulating NFIB activity, perhaps via NFIB interaction partners, such as endogenous NFIs.

PIN1 increases NFIB's stability

Next, as PIN1 often increases or decreases the stability of its substrates, we asked if such an effect on NFIB's half-life can be another consequence of PIN1

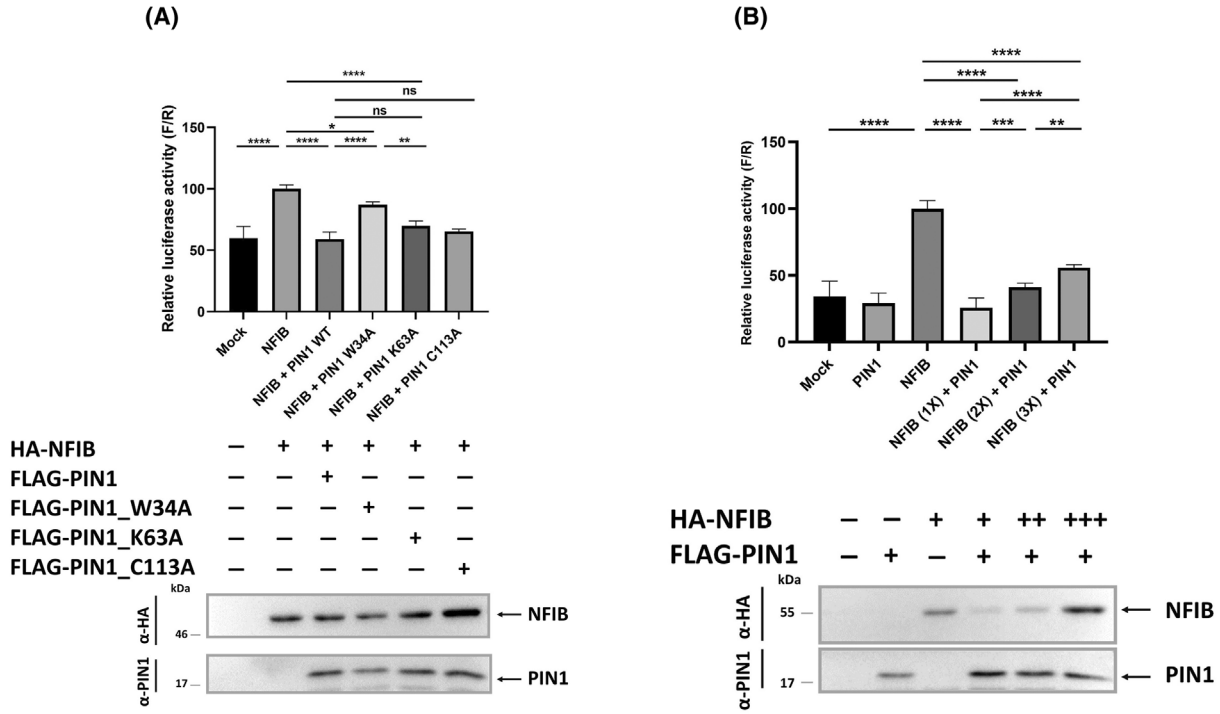

Fig. 6. PIN1 represses NFIB-induced *GFAP-Luc* promoter activity. (A) Transcriptional activity of NFIB was monitored via luciferase assays. HEK293T cells were transfected with HA-NFIB, FLAG-PIN1, or FLAG-PIN1 mutant expression plasmids together with *GFAP-luciferase* and *Renilla-luciferase* plasmids in 24 well plates. Forty-eight hours after the transfection, cells were harvested and the firefly luciferase activity was measured and normalized to renilla luciferase activity. (B) HEK293T cells were transfected with increasing amounts of HA-NFIB expression plasmids as described in (A). Amount of overexpressed HA-NFIB and PIN1 proteins was monitored by WB analysis. Luciferase assays were carried out in triplicate. Data are expressed as mean ± SD of normalized luciferase activity and shown as fold change relative to those of HA-NFIB transfected cells. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$; ns, not significant. Statistical significance was determined using one-way Anova. Results are representative of three independent experiments.

interaction. To address this possibility, we overexpressed Flag-PIN1 along with HA-NFIB in HEK293T cells and treated the cells with cycloheximide (CHX) to block new protein synthesis. We found that by 8 h of CHX treatment, NFIB levels were significantly higher in the presence of overexpressed PIN1 (Fig. 7A,B). Overexpression of NFIB interaction-deficient PIN1 mutant (W34A) did not affect NFIB levels, while PIN1-K63A acted similarly to wild-type PIN1, increasing HA-NFIB's stability (Fig. S5). Moreover, when PIN1 levels were reduced using PIN1 targeting small hairpin RNA encoding lentiviruses, half-life of HA-NFIB decreased relative to control (Fig. 7C,D). While the decrease was not statistically significant, a trend towards reduced HA-NFIB half-life was observed in PIN1-depleted cells over multiple experiments. Taken together, these results strongly suggest that apparently by inducing a conformational change in NFIB, PIN1 decreases its transcriptional activity while increasing its stability. PIN1's effect on NFIB transcriptional activity may also involve dimers of NFIB with endogenously expressed NFI family members which appear to bind PIN1 with similar affinity *in vitro*.

Discussion

Nuclear factor one transcription factors are required for proper differentiation of neural progenitors during CNS development [8] and specifically, maintenance of core transcriptional programs in brain cancer cells, such as glioblastoma [10]. Meanwhile, NFIs, and particularly NFIB, have been assigned both tumor suppressor and oncogenic roles, even in the same type of tumor [7,75–77]. NFI family members may carry out their divergent functions in oncogenic pathways via interaction of their diverse C-terminal domain with regulatory proteins. A Y2H screen identified PIN1 as such a potential regulator of NFIB. Indeed, PIN1 binds to the well-conserved N-terminal DBD domain

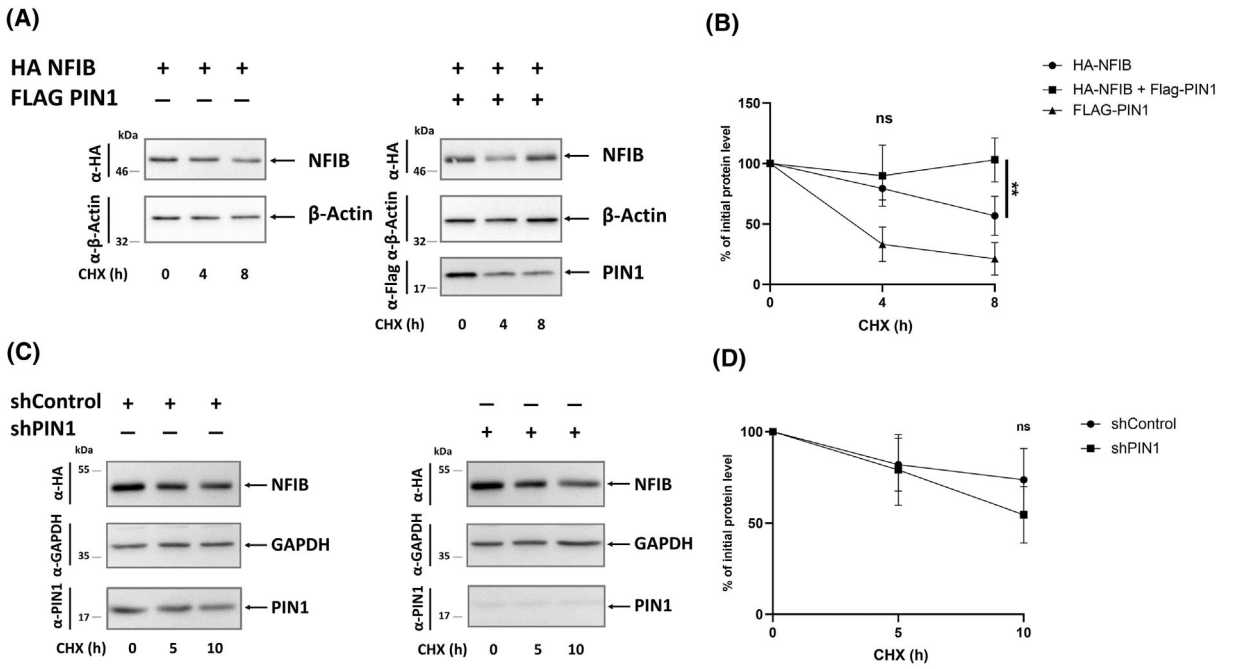

Fig. 7. Overexpression of PIN1 increases the half-life of HA-NFIB. (A) For overexpression experiments, HEK293T cells were transfected with HA-NFIB and Flag-PIN1. Forty-eight hours after the transfection, cells were treated with 50 $\mu g \cdot mL^{-1}$ CHX for the indicated times. A representative result from three independent experiments is shown. (B) Quantification of the band intensities of three independent experiments. (C) For knockdown experiments, HEK293T cells were infected with shPIN1 or shControl encoding lentiviral particles and the next day the cells were transfected with HA-NFIB. Forty-eight hours after the transfection, cells were treated with 50 $\mu g \cdot mL^{-1}$ CHX for indicated times. A representative result from three independent experiments is shown. (D) Quantification of the band intensities of three independent experiments. Statistical significance was determined using two-way ANOVA with Sidak's multiple comparisons test, data are represented as mean ± SEM, $^{**}P < 0.01$; ns, not significant.

of NFIB as well as other NFI family members. Moreover, PIN1 represses NFIB's transcriptional activity while seemingly increasing NFIB's abundance.

Nuclear factor one transcriptional activity is reportedly subject to post-translational regulation by phosphorylation [78–80] rendering NFIB a potential target for PIN1, which isomerizes phosphorylated motifs on its targets leading to profound effects on signaling pathways [42]. Indeed, the data suggest that PIN1-NFIB interaction requires phosphorylation of NFIB, since CIP-dependent dephosphorylation of NFIB significantly weakened NFIB-PIN1 binding. Although PIN1 can bind non-canonical motifs on its target proteins [72,81,82], it preferentially recognizes the phosphorylated Ser/Thr-Pro sequences [42,74,83]. Moreover, mutating all conserved eight Ser/Thr-Pro motifs to Ala-Pro in NFIB appeared not to affect NFIB's affinity for PIN1 (Fig. S4B–D). Furthermore, PIN1 binds to the N-terminal DBD domain of NFIB, specifically to the region spanning residues 167–208. Interestingly, NFIB N-terminal DBD domain has only two typical PIN1 binding motifs (Ser4-Pro and Ser194-Pro); thus the region 167–208 harbors only the one site (Ser194-Pro). However, mutagenesis of both motifs on the full-length protein does not disrupt NFIB-PIN1 interaction (Fig. S4B–D). Moreover, NFI N-terminal DBD domains carry additional conserved non-canonical PIN1 motifs potentially subject to phosphorylation (e.g. Ser-Ser, Ser-Gly) [84–88] as well as proline residues that can also act as PIN1 binding motifs (Fig. S3) [72,89]. It is possible that these non-canonical sites on the DBD domain serve as PIN1 interaction motifs. Recent evidence indicates that PIN1 may bind to multiple domains of its substrates [88]. Finally, these findings cannot overrule weak interactions by the C-terminal region; proline-rich regions on the C-terminal TAR domain, while not sufficient for, may also contribute to overall NFI affinity for PIN1. Nevertheless, further experiments are required to characterize the PIN1 binding motif(s) on NFIB to fully understand how the interaction occurs at the biochemical level.

We found that the NFIB N-terminal DBD domain is necessary and sufficient for PIN1 binding, whereas

NFIB C-terminal TAR domain, which is less conserved among NFI family members, does not bind to PIN1. As NFI DBD domains are 80–90% homologous [12], expectedly, the other NFI family members, NFIA, NFIC, and NFIX, also interact with PIN1 via their N-terminal domains. This suggests that PIN1 may regulate transcriptional activity and abundance of other NFIs, as well as NFIB. However, PIN1 may specifically interact with and differentially regulate each family member, as NFIs display distinct expression patterns during embryonic development as well as in the adult [90,91].

Until recently, only a number of proteins, mostly transcription factors such as FOXA1, SKI, OLIG2, SOX9 have been reported to interact with NFI family members and co-regulate gene expression [34,36–38]. Subsequently, a screen identified a multitude of protein–protein interactions involving transcription factors, most of which presumably participate in gene regulatory networks with NFI proteins [39]. In contrast, PIN1 controls substrate transcription factors, modulating their localization, abundance, and conformation. To test for PIN-dependent regulation of NFIB function, we utilized activation of *GFAP* promoter, a well-characterized NFI target [15]. We found that co-expression of PIN1 with NFIB suppresses NFIB's transcriptional activity. As PIN1 WW domain mutations disturb PIN1-NFIB interaction while PPIase null mutations have no effect (Fig. 5A–C), we also tested these mutants in *GFAP* promoter activation experiments. Indeed, W34A mutation eliminated PIN1's suppression of NFIB, whereas PIN1-K63A or PIN1-C113A was still a potent inhibitor of NFIB-induced *GFAP* transcription. Thus, we posit that PIN1 acts as a repressor to hinder NFIB's transcriptional activity, and the isomerization by PIN1 is not required for this inhibition. In general, the catalytic activity of PIN1 has been shown to be necessary for its regulatory effects [46,70,92]; however, the PPIase activity-independent actions of PIN1 have been also reported for several target proteins [55,88,93,94]. Moreover, catalyzed isomerization may be only one of the consequences, and not always a relevant one, of PPIase interactions [95]. Interestingly, in the presence of over-expressed PIN1, even high levels of NFIB could not recover the levels of *GFAP* promoter activation induced by NFIB alone. These data suggest that PIN1 executes its repressive effect on NFIB's transcriptional activity not via regulating its abundance but by inducing a conformational change, through a mechanism that is not yet characterized. Alternatively, PIN1 could be facilitating assembly of transcription regulatory multiprotein complexes which may or may not involve other NFI family members. Future experiments that utilize pharmacological inhibition of PIN1's PPIase activity would help elucidate the mechanisms involved.

While we found that PIN1-NFIB interaction results in weakened NFIB transcriptional activity, our findings also support PIN1's augmentation of NFIB protein stability (Fig. 7A–D). Interestingly, PIN1's seemingly paradoxical effects have been reported for other transcription factors, such as c-MYC, and SP1 [69,96]. PIN1 was shown to increase recruitment of phosphorylated c-MYC to target gene regulatory regions while directing c-MYC towards proteasomal degradation [96]. In contrast, PIN1 enhances stability of SP1 by interfering with phosphorylated SP1-ubiquitin E3-ligase interaction even as reducing its DNA binding activity, leading to its complete removal from chromatin [69]. Therefore, we surmise that binding by PIN1 may change the conformation of NFIB without isomerizing it, which in turn changes NFIB's interaction with other proteins to enhance transcription while perhaps reducing its affinity for proteasome degradation machinery. The exact mechanism of NFIB regulation by PIN1 remains to be further characterized.

We found that endogenous PIN1 binds to NFI proteins in SH-SY5Y neuroblastoma cells. While subcellular localization of PIN1 can vary with cell and tissue types [97], it has been reported to be localized to the nucleus in SH-SY5Y cells [98]. *In vivo*, during CNS development, PIN1, NFIA, NFIB, and NFIX are all expressed by neural progenitor cells (NPCs). As NFIs are strictly localized to the nucleus [99] while PIN1 expression has been detected in both nuclei and cytoplasm of cultured NPCs and neurons [40,44] and the PIN1-NFI proteins are likely to interact. Neuronal differentiation and proliferation of *Pin1*$^{-/-}$ NPCs is decreased *in vitro*, and upper layers of *Pin1*$^{-/-}$ cortex display neurogenesis defects *in vivo* while gliogenesis is apparently unaffected [40,44]. In contrast, NFIB, along with NFIA and NFIX, regulate differentiation of neural progenitors into glia as well as neurons, induce glial effector genes, including the target gene *GFAP*, as well as genes required for terminal differentiation of neurons [8]. Therefore, it would be interesting to characterize *in vivo* expression of NFIs and PIN1 in more detail, as potential regulation of NFIB by PIN1 may depend on co-localization in differentiating progenitors, at specific developmental time points and cellular compartments.

In addition to their roles during the development of CNS, both PIN1 and NFIB are misregulated in CNS tumors. For instance, in glioblastoma, PIN1 expression levels increase while NFIA/B levels are also

altered in particular glioblastoma subtypes [10,51,54,75,76]. Interestingly, expression of *GFAP*, an important terminal differentiation effector gene, is significantly low in malignant glioma cells and even completely lost with increasing tumor grade [100,101]. Therefore, as GFAP levels are associated with better survival, PIN1-dependent suppression of NFIB's transcriptional activity may lead to downregulation of *GFAP* and may be involved in tumor progression. However, elucidation of PIN1's effect on NFIB-GFAP axis in cancer cells awaits further investigation.

In conclusion, we demonstrate that PIN1 is a novel NFIB interaction partner which, via its WW domain, binds to NFIB in a phosphorylation-dependent manner. PIN1 has dual effects on NFIB, as it represses transcriptional activation of *GFAP* by NFIB while increasing its abundance. Although yet unexplored, PIN1 has the potential to functionally regulate the other NFI family members as the interaction requires NFIs' conserved DBD domains. Since both proteins are misregulated in tumor cells and are functionally linked to tumor progression, it would be interesting to uncover downstream consequences of PIN1-NFIB interaction and to determine whether it is subject to regulation by oncogenic signaling pathways.

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Author contributions

AK designed the study, received funding for the project, supervised the research process, and edited the manuscript. SSE contributed to the study design and conception, performed most of the experiments, analyzed the data, wrote the original manuscript, and contributed to the funding of the study. AEY performed the yeast two-hybrid assay.

Peer review

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1002/1873-3468.15010.

Data accessibility

The data that support the findings of this study are available in Figs 1–7 and the Supporting Information of this article.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Comparison of endogenous versus overexpressed HA-NFI expression levels in HEK293T cells.
Fig. S2. Endogenous PIN1 interacts with NFIs.
Fig. S3. NFI protein sequence alignment.
Fig. S4. Site-directed mutagenesis of NFIB Ser/Thr-Pro motifs does not abolish PIN1 interaction.
Fig. S5. Overexpression of wild-type PIN1 and PIN1-K63A increases the half-life of HA-NFIB whereas PIN1-W34A has no effect.