

Impaired dynamin 2 function leads to increased AP-1 transcriptional activity through the JNK/c-Jun pathway



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ABSTRACT

Activation of AP-1 transcription factors, composed of the Jun and Fos proteins, regulates cellular fates, such as proliferation, differentiation or apoptosis. Among other stimuli, the AP-1 pathway can be initiated by extracellular ligands, such as growth factors or cytokines, which undergo internalization in complex with their receptors. Endocytosis has been implicated in the regulation of several signaling pathways; however its possible impact on AP-1 signaling remains unknown. Here we show that inhibition of dynamin 2 (Dyn2), a major regulator of endocytic internalization, strongly stimulates the AP-1 pathway. Specifically, expression of a dominant-negative Dyn2 K44A mutant increases the total levels of c-Jun, its phosphorylation on Ser63/73 and transcription of AP-1 target genes. Interestingly, *DNM2* mutations implicated in human neurological disorders exhibit similar effects on AP-1 signaling. Mechanistically, Dyn2 K44A induces AP-1 by increasing phosphorylation of several receptor tyrosine kinases. Their activation is required to initiate a Src- and JNK-dependent signaling cascade converging on c-Jun and stimulating expression of AP-1 target genes. Cumulatively, our data uncover a link between the Dyn2 function and JNK signaling which leads to AP-1 induction.

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1. Introduction

Signaling via activator protein-1 (AP-1) transcription factors can control a wide range of cellular processes, including proliferation, differentiation, migration, survival or death. In turn, deregulation of the AP-1 pathway has been implicated in several types of pathological conditions, such as developmental disorders, inflammation and cancer [1–3]. The AP-1 proteins belong to three main subfamilies: Jun (c-Jun, JunB, JunD), Fos (c-Fos, FosB, Fra-1, Fra-2) and ATF [4,5]. All members contain a conserved basic region-leucine zipper (bZIP) domain, responsible for dimerization and DNA binding at the TPA-responsive elements (TREs; 5'-TGAG/CTCA-3'). Individual AP-1 proteins differ with respect to their dimerization and transactivation abilities. Members of the Jun subfamily can homo- and heterodimerize, while Fos or ATF proteins cannot form homodimers [2]. The transactivation ability of c-Jun, c-Fos and FosB is strong, relative to the weak activities of JunB, JunD or Fra-1/2. The latter proteins can also act as transcriptional repressors by heterodimerizing with strong activators [5–7].

Signaling that activates AP-1 complexes can be initiated by growth factors, cytokines, stress conditions or phorbol esters. It proceeds via MAP kinase (MAPK)-based cascades engaging c-Jun N-terminal kinases

(JNKs) or extracellular signal-regulated kinases (ERKs) [5]. A typical sequence of activation involves: protein kinase C (PKC), MAP kinase kinase (MEKK), MAPK kinase (MKK) and JNK. The latter induces phosphorylation of c-Jun on Ser63 and Ser73, leading to formation of active AP-1 dimers [8]. Although the AP-1 pathway can be induced by extracellular ligands which bind appropriate plasma membrane receptors and undergo internalization, it remains poorly investigated how endocytosis affects the AP-1 signaling outcome.

Endocytosis, initially viewed merely as a transport route leading to degradation or recycling of extracellular cargo, is now known to contribute to various aspects of cell signaling at multiple levels [9–11]. Among other functions, endocytosis regulates a number of receptors at the plasma membrane, thus affecting the overall cell responsiveness to specific ligands [12]. Several internalization routes are dependent on dynamins which are large GTPases involved in budding and scission of clathrin-coated vesicles and of other endocytic intermediates from the plasma membrane [13,14]. Endocytosis mediated by dynamin can be blocked by pharmacological inhibition of its GTPase activity [15,16] or by overexpression of a dominant-negative mutant that contains a K44A amino acid substitution in the nucleotide-binding site [17]. Impaired GTP binding and hydrolysis by the K44A mutant prevents proper structural reorganization of dynamin oligomers crucial for membrane fission [18], thereby inhibiting scission of endocytic vesicles and cargo internalization as shown for various receptors in several cell types [17,19,20].

In mammals, there are three dynamin-encoding genes, of which dynamin 2 is ubiquitously expressed. Its inactivation in mouse causes

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early embryonic lethality [21], while mutations in the human *DNM2* gene lead to rare forms of the Charcot–Marie–Tooth peripheral neuropathy (CMT) and autosomal dominant centronuclear myopathy (CNM) [22–25]. The vast majority of disease-associated *DNM2* mutations are clustered in the middle part of the protein, comprising the pleckstrin homology (PH) and GTPase effector (GED) domains. They exhibit dominant-negative effects which may result from perturbations of endocytosis or microtubule-related functions [24,26]. Nevertheless, the pathomechanism of both diseases is still an open question and probably results from impairment of different dynamin-mediated processes [27].

Here we demonstrate that inhibition of dynamin activity drives AP-1 dependent signaling, reflected by increased activation of JNK, c-Jun and enhanced target gene expression. Importantly, disease-associated *DNM2* mutations cause similar effects on the AP-1 pathway. At the molecular level, increased AP-1 signaling results from enhanced activation of several growth factor receptors which is observed upon dynamin inhibition.

2. Materials and methods

2.1. Plasmids

The following plasmids were used: mouse HA-tagged dynamin 2 K44A construct (HA-mDyn2 pcDNA3) was a kind gift from Dr. P. de Camilli (Yale University School of Medicine, USA); human WT Cherry-tagged dynamin 2 construct (Dyn2-pmCherry-N1) was provided by Dr. Ch. Merrifield (Addgene plasmid #27689, Addgene, Cambridge, MA, USA); A-Fos construct was provided by Dr. Ch. Vinson (Addgene plasmid #33353). For luciferase assays we used the following plasmids: pAP-1-luc from Stratagene (Santa Clara, CA, USA), pRL-TK from Dr. Vladimir Korinek (Institute of Molecular Genetics, Prague, Czech Republic) and pTA-Luc from Clontech (Mountain View, CA, USA). Plasmid encoding human TYRO3 (SC108283) was from Origene (Rockville, MD, USA). Plasmids encoding EGFR were from Dr. I. Dikic (Goethe University Frankfurt, Germany). Plasmids encoding untagged or HA-tagged PDGF receptor were constructed by cloning cDNA of *PDGFRB* into pcDNA vector.

Mutations in human *DNM2* were introduced using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) at positions: K44A, R465W, K562E, V625 del and E650K in Dyn2-pmCherry-N1. All constructs were verified by sequencing.

2.2. Antibodies and chemicals

The following antibodies were purchased from Santa Cruz Biotechnology (Dallas, Texas, USA): anti-phospho-c-Jun Ser63/73 (sc-16312), anti-c-Jun (sc-45), anti-JunB (sc-8051), anti-JunD (sc-74), anti-cFos (sc-52), anti-phospho-cFos Thr 232 (sc-130181), rabbit anti-PDGFR- β (sc-432), anti-GAPDH (sc-25778) and anti-lamin A/C (sc-7292). Anti-TYRO3 (ab109231) and anti-RFP (#62341) antibodies were from Abcam (Cambridge, UK), anti-phosphotyrosine (pTYR) (05-321) from Merck Millipore (Darmstadt, Germany) and anti-dynamin (610245) from BD Transduction Laboratories (Franklin Lakes, NJ, USA). Anti-phospho-SAPK/JNK (Thr183/Tyr185) (#9255), anti-SAPK/JNK (#9252), anti-ERK1/2 (#9107) and anti-phospho-ERK1/2 (p44/42 MAPK; Thr202/Tyr204) (#4370) were from Cell Signaling (Danvers, MA, USA). The following secondary antibodies were used: horseradish peroxidase-conjugated (#115-035-062, #805-035-180 and 111-035-144; Jackson ImmunoResearch; West Grove, PA, USA) and fluorophore-conjugated (#926-680-23 and #926-32212; LI-COR Biosciences, Lincoln, NE, USA). All primary antibodies were used at dilution of 1:500 or 1:1000 for immunoblotting. All secondary antibodies were used at dilution of 1:10,000.

Transferrin labeled with Alexa Fluor®647 was from Invitrogen (Waltham, MA, USA). Dynamin inhibitor Dynole 34-2 and its inactive control, Dynole 31-2, were from Ascent Scientific (now Abcam Biochemicals, Bristol, UK). PMA and Src kinase inhibitors SU6656 and

SKI-1 were from Sigma-Aldrich (St. Louis, MO, USA). JNK inhibitors, SP600125 and JNK inhibitor VIII, were from Merck Millipore (Darmstadt, Germany). RTK inhibitors Canertinib and SU-11274 were from BioVision (Milpitas, CA, USA). The inhibitors were used in the following concentrations: 20 μ M Dynole 34-2; 20 μ M Dynole 31-2; 2.5 μ M SU6656; 2.5 μ M SKI-1; 3 μ M Canertinib and 10 μ M SU-11274. PMA was used at 50 μ M concentration. DMSO (Bioshop, Burlington, Canada) served in an equivalent volume as a control and solvent for all inhibitors, Dynole 31-2 and PMA.

2.3. Cell culture

HEK293 (human embryonic kidney 293) cells were maintained in DMEM (Dulbecco's modified Eagle's medium, Sigma-Aldrich) supplemented with 10% FBS and 2 mM L-glutamine (Sigma-Aldrich).

2.4. Serum starvation

In experiments performed with serum-starved cells, medium was exchanged to DMEM containing 0.2% BSA 18 h prior to lysis.

2.5. Transferrin uptake

Cells pretreated for 30 min with 2.5 μ M dynamin inhibitor (Dynole 34-2), its analog (Dynole 31-2) or DMSO were exposed to Alexa Fluor®647-labeled transferrin (20 μ g/ml) for 15 min. at 37 °C. Afterwards they were placed on ice and washed two times with ice-cold PBS, then once with ice-cold DMEM without FBS with pH adjusted to 3 and finally once with PBS. Cells were lysed in RIPA buffer and extracts subjected to Western blotting. Bands of fluorescently labeled transferrin were detected and quantified in Odyssey infrared imaging system, normalized to EEA1 protein.

2.6. Cell transfection

Transient DNA transfections were done using Lipofectamine 2000 (Life Technologies, Paisley, UK) according to manufacturer's instructions and analyzed 24 h after transfection (48 h for qPCR analysis). For luciferase assays, one day before transfection 1.7×10^4 cells were seeded per well in a 96-well dish, for qRT-PCR analysis 1.6×10^5 cells were seeded per well in 12-well dish and $2.5\text{--}3 \times 10^6$ cells were seeded in 100 mm dish for nuclear extraction and for Phospho-RTK array analysis.

2.7. Luciferase assay

For luciferase assay HEK293 cells were transfected in 96-well plates with 50 ng of reporter (pAP-1-luc) and 10 ng of normalization construct (pRL-TK) along with 25 ng of Dyn2-encoding plasmids or with empty vector (pcDNA). Four transfections were performed in parallel and repeated in at least three independent experiments. 24 h posttransfection cells were lysed in Passive Lysis Buffer (Promega, Madison, WI, USA) and either luciferase assay or immunoblotting was performed. For experiments with inhibitors, 5 h post-transfection cells were treated with medium containing inhibitor or control (DMSO) and cultured for further 19 h. In cell lysates, the activity of luciferases was measured using the following buffers: Firefly Buffer [50 mM Tris pH 7.8, 12 mM MgCl₂, 10 mM DTT, 0.2 mM ATP (Sigma-Aldrich), 0.5 mM D-luciferin (LuxBiotechnology, UK), 0.25 mM coenzyme A (Sigma-Aldrich)] or Renilla Buffer [50 mM Tris pH 7.8, 100 mM NaCl, 2.5 nM coelenterazine (Lux Biotechnology)] and microplate luminometer (Centro XS3 LB 960, Berthold Technologies, USA). The firefly luciferase signal was normalized to its respective Renilla luciferase readout. Measurements are presented as the fold change relative to the pathway activity in cells transfected with control vector. Values correspond to the mean \pm

s.e.m. from at least three independent experiments, each with three or four independent transfections performed in parallel.

2.8. Quantitative RT-PCR

Expression of selected AP-1 target genes was verified at 48 h after transfection of HEK293 cells with the plasmids encoding WT or mutated Dyn2. In some experiments 18 h before lysis, cell medium was exchanged for DMEM not supplemented with serum. For RNA isolation and cDNA synthesis, GenElute Mammalian Total RNA Miniprep (Sigma-Aldrich), random nonamers, oligo(dT)23 and M-MLV reverse transcriptase (Sigma-Aldrich) were used according to manufacturer's instructions. For all qRT-PCR reactions TaqMan probes, TaqMan® Gene Expression Master Mix (both from Life Technologies) and 7900HT Fast Real-Time PCR thermocycler (Applied Biosystems, Waltham, MA USA) were used. qPCR data were analyzed by the $\Delta\Delta C_t$ method implemented in the DataAssist software v 3.01 (Applied Biosystems). The data were normalized according to the level of *GAPDH* and *B2M* (encoding glyceraldehyde 3-phosphate dehydrogenase and beta-2-microglobulin, respectively). All values are relative expression levels (fold change) compared to controls from cells transfected with an empty plasmid (normalized to 1 for each gene).

2.9. Cell fractionation and DNA-binding ELISA assay

HEK293 cells were transfected in 100 mm dishes with appropriate plasmids for 24 h, harvested and fractionated as described previously [28]. In order to shear the DNA, nuclear samples were spun through QIAshredder columns (Qiagen, Hilden, Germany). Samples of nuclear fractions containing 10–20 μg protein were assayed using the TransAM® AP-1 Family kit according to the manufacturer's instructions (Active Motif, Carlsbad, CA, USA). Samples of 50 μg protein were used for Western blotting.

2.10. Western blotting and densitometry analysis

To obtain whole cell lysates, HEK293 cells were lysed in RIPA buffer containing 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris (pH 7.4), 150 mM NaCl, 0.5 mM EDTA, protease inhibitor cocktail (6 $\mu\text{g}/\text{ml}$ chymostatin, 0.5 $\mu\text{g}/\text{ml}$ leupeptin, 10 $\mu\text{g}/\text{ml}$ antipain, 2 $\mu\text{g}/\text{ml}$ aprotinin, 0.7 $\mu\text{g}/\text{ml}$ pepstatin A and 10 $\mu\text{g}/\text{ml}$ 4-aminodiphenylmethanesulfonyl fluoride hydrochloride; Sigma-Aldrich) and phosphatase inhibitor cocktails (Sigma-Aldrich). Protein concentration was measured with BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Samples of 10–50 μg total protein were subjected to SDS-PAGE. Resolved proteins were transferred to nitrocellulose membrane (Amersham Protran, 0.44 μm NC, GE Healthcare Life Sciences, UK), probed with specific antibodies, and detected with ECL chemiluminescence reagent on ImageQuant LAS 4000 (GE Healthcare Life Sciences) or Odyssey infrared imaging system (LI-COR Biosciences). ImageJ program was used to measure the density of Western blot bands from cell fractionation experiments. Each single value was calculated by dividing a signal of the analyzed protein by a signal from lamin A/C content in the sample.

2.11. Human Phospho-RTK array

Human Phospho-RTK array (R&D Systems, Minneapolis, MN, USA) was used according to the manufacturer's instructions. Briefly, cells were lysed on ice in the provided lysis buffer. 700 μg or 800 μg of lysates was incubated with blocked array membranes overnight, washed and incubated with horseradish peroxidase-conjugated anti-phosphotyrosine for 2 h at room temperature. The membranes were washed again before development with ECL Western blotting detection reagent provided by the manufacturer. The signals were densitometrically quantified with ImageJ software. The amounts of phosphorylated RTKs were compared

to levels present in control cells (non-transfected serum-grown or pcDNA-transfected) which were normalized to 1.

2.12. Statistical analysis

Statistical analyses were carried out using the software STATISTICA 8.0 (StatSoft). Normality of data distribution was assessed using the Shapiro–Wilk test. For luciferase assay data, raw values (before normalization) from the control and examined groups were compared using nonparametric Mann–Whitney U test. One-sample t-test was applied for normalized data from qPCR or Western blot densitometry analysis. For normally distributed data independent-samples t-test was applied. Differences were considered statistically significant for $P \leq 0.05$.

3. Results

3.1. Impairment of dynamin 2 GTPase activity potentiates AP-1 dependent transcription

We used an established luciferase reporter harboring AP-1 binding sites [29] to investigate the role of dynamin 2 in the regulation of AP-1 dependent transcription. Basal AP-1 activity was measured in serum-grown HEK293 cells, upon overexpression of a dominant-negative K44A mutant of dynamin 2 (Dyn2 K44A). We found that the presence of the K44A mutant increased AP-1 mediated transcription of luciferase reporter (Fig. 1A, pAP-1-luc). This was linked to the disrupted GTPase activity of Dyn2 assemblies, rather than an increased level of the protein itself, because cells overexpressing the wild-type (WT) Dyn2 showed unchanged AP-1 activation. To confirm that observed alterations in reporter gene expression reflected specifically the AP-1 activity, we analyzed the impact of overexpressed WT or K44A Dyn2 on the expression levels of a control luciferase reporter lacking AP-1 binding sequences in the promoter site (Fig. 1A, pTA-luc reporter). As expected, the luciferase gene expression remained at control levels in both Dyn2 WT and K44A-producing cells. As an additional specificity control, we employed A-Fos, a mutated c-Fos protein which acts as dominant negative for c-Jun and abolishes AP-1 binding to DNA [30]. Indeed, expression of A-Fos down-regulated both basal and Dyn2 K44A-induced AP-1 activity (Fig. 1B).

To confirm that the effect of K44A overexpression on AP-1 was due to the impaired activity of endogenous Dyn2, we employed a pharmacological inhibition approach. Consistently, treatment of cells with Dynole 34-2, a dynamin GTPase inhibitor [31], activated the AP-1 reporter in a concentration-dependent manner in comparison to solvent-treated cells (Fig. 1C). This was not observed upon administration of an inactive analog of the inhibitor, Dynole 31-2. Within the range of tested concentrations, Dynole 34-2 inhibited dynamin-mediated endocytosis, as verified in a transferrin uptake assay (Fig. S1A) and consistent with reports for other cell lines [15,32]. Cumulatively, these results indicated that the Dyn2 GTPase activity is involved in the negative regulation of AP-1 transcriptional activity.

3.2. Dyn2 K44A increases levels of c-Jun and induces its Ser63/73 phosphorylation

AP-1 transcriptional activity can result from increased abundance of AP-1 subunits in the nucleus or from their post-translational modifications which enhance their dimerization and DNA-binding abilities [33]. To examine which of these mechanisms could underlie the increase of the AP-1 reporter activity upon Dyn2 inhibition, we measured the nuclear levels of AP-1 subunits: c-Jun, JunB, JunD and c-Fos (they were hardly detected in non-nuclear fractions in control or in Dyn2 K44A-expressing cells; Fig. S1B). We observed that Dyn2 K44A expression increased the levels of c-Jun in the nucleus by nearly 40% without affecting the abundance of JunB and JunD (Fig. 2A, immunoblots and quantification on the right). Moreover, the elevated c-Jun was strongly phosphorylated on Ser63/73. The overall amounts of phosphorylated

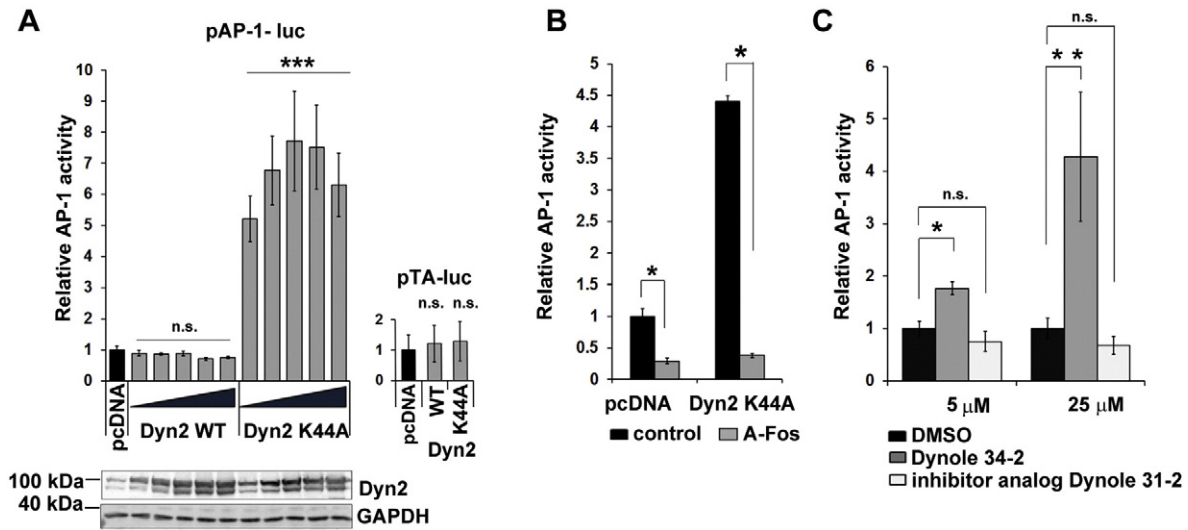


Fig. 1. Dynamin 2 GTPase activity is involved in AP-1 regulation. (A) Expression of GTPase-deficient Dyn2 K44A mutant increases the activity of AP-1 dependent luciferase reporter (pAP-1-luc) in unstimulated serum-grown HEK293 cells. The reporter activity in cells transfected with an empty vector (pcDNA) was normalized to 1. Increasing amounts (5–100 ng/well of 96-well plate) of Dyn2 K44A-encoding plasmid were used for transfections. No effects were observed in cells overexpressing WT Dyn2 or in cells expressing AP-1 independent luciferase reporter (pTA-Luc, transfected along with 25 ng of dynamin-encoding plasmids). The levels of Dyn2 WT and K44A overexpression were verified by Western blotting, with GAPDH serving as a loading control (lower panel). (B) Inhibition of c-Jun by dominant negative A-Fos prevents AP-1 activation by Dyn2 K44A. Data were normalized to the luciferase activity in pcDNA-transfected control cells. (C) Inhibition of endogenous Dyn2 by Dynole 34-2 increases the AP-1 reporter activity. Dynole 31-2 (an inactive analog) was used as control. The reporter activity in cells treated with inhibitor vehicle (DMSO) was normalized to 1. (A–C) All data are mean \pm s.e.m. from 3 to 5 independent experiments; * $P \leq 0.05$, ** $P < 0.01$ (Mann–Whitney U test).

c-Jun increased 4.5-fold in comparison to basal levels in control cells. It is known that phosphorylation of Ser63/73 enhances protein stability and transcriptional activity of c-Jun homo- and heterodimers [8,34] that could contribute, at least partially, to elevated levels of total c-Jun in the nucleus.

In contrast to Jun family members, basal levels of c-Fos are low or undetectable in most cell types. Various stimuli (e.g. serum) may induce its rapid, although transient, expression and phosphorylation [4]. In line with this, high levels of c-Fos protein and its active Thr232-phosphorylated form were detected in lysates from cells treated with phorbol 12-myristate 13-acetate (PMA), a strong AP-1 activator, but not in unstimulated cells transfected with an empty vector or plasmids encoding Dyn2 WT or K44A (Fig. 2B). Based on these data, we concluded that in HEK293 cells c-Fos is not involved in basal or Dyn2 K44A-induced AP-1 activity.

Although the total amounts of JunB and JunD were not changed upon Dyn2 K44A expression, we could not exclude their contribution to the increased AP-1 activity. There are no clearly defined phosphorylation sites within JunB and JunD sequences that could serve as markers for their transcriptional activity. Since Jun proteins can homo- and heterodimerize with each other, we used an ELISA-based method to verify the contribution of individual AP-1 members to the increased activity in Dyn2 K44A-expressing cells. In this test, immobilized oligonucleotides containing a TRE motif are employed to capture AP-1 dimers and individual constituents of these complexes are quantified by specific antibodies. Specificity of AP-1-DNA interaction is tested in the presence of wild-type or mutated competitor oligonucleotides (the latter should not affect the signal strength). As shown in Fig. 2C, Dyn2 K44A-expressing cells contained higher amounts of c-Jun phosphorylated on Ser63 bound to the TRE-containing oligonucleotides, in agreement with the Western blot data (Fig. 2A). However, DNA-bound AP-1 dimers contained also JunB and JunD, although their enrichment was lower than for phospho-c-Jun. It is therefore plausible that Dyn2 K44A expression also indirectly increases the DNA-binding activity of JunB and JunD, without affecting their total amounts in the cell but possibly by their dimerization with active Ser63/73-phosphorylated c-Jun monomers. Finally, in this assay we did not detect any active proteins from the Fos subfamily (c-Fos, FosB, Fra-1) in control

or Dyn2 K44A-expressing cells (data not shown). This underscores our initial conclusion that the Fos proteins do not participate in AP-1 activation induced by loss of the Dyn2 GTPase activity.

3.3. Dyn2 K44A induces expression of AP-1 target genes

AP-1 modulates expression of multiple target genes but their spectrum and the amplitude of change differ depending on the AP-1 dimer composition and the cell type [35]. We investigated whether Dyn2 K44A-induced activation of c-Jun correlated with increased expression of known AP-1 target genes. One of them is the *JUN* gene itself because c-Jun is a positive regulator of its own transcription [36]. This autoregulation of c-Jun appears to be unique among AP-1 transcription factors and has been shown to prolong and amplify transient signals generated by extracellular stimuli [37,38]. First, we compared the *JUN* and *JUNB* mRNA levels in cells expressing WT and mutated version of Dyn2. We found >2-fold increase of *JUN* expression in cells with perturbed dynamin activity (Fig. 2D). These data were in accordance with Western blot results (Fig. 2A). Higher expression of *JUN* may contribute to the AP-1 transcriptional activation by Dyn2 K44A (Fig. 1A). At the same time, the *JUNB* mRNA level was comparable to control cells (Fig. 2D), indicating that JunB is not regulated transcriptionally or translationally (Fig. 2A) upon Dyn2 K44A expression.

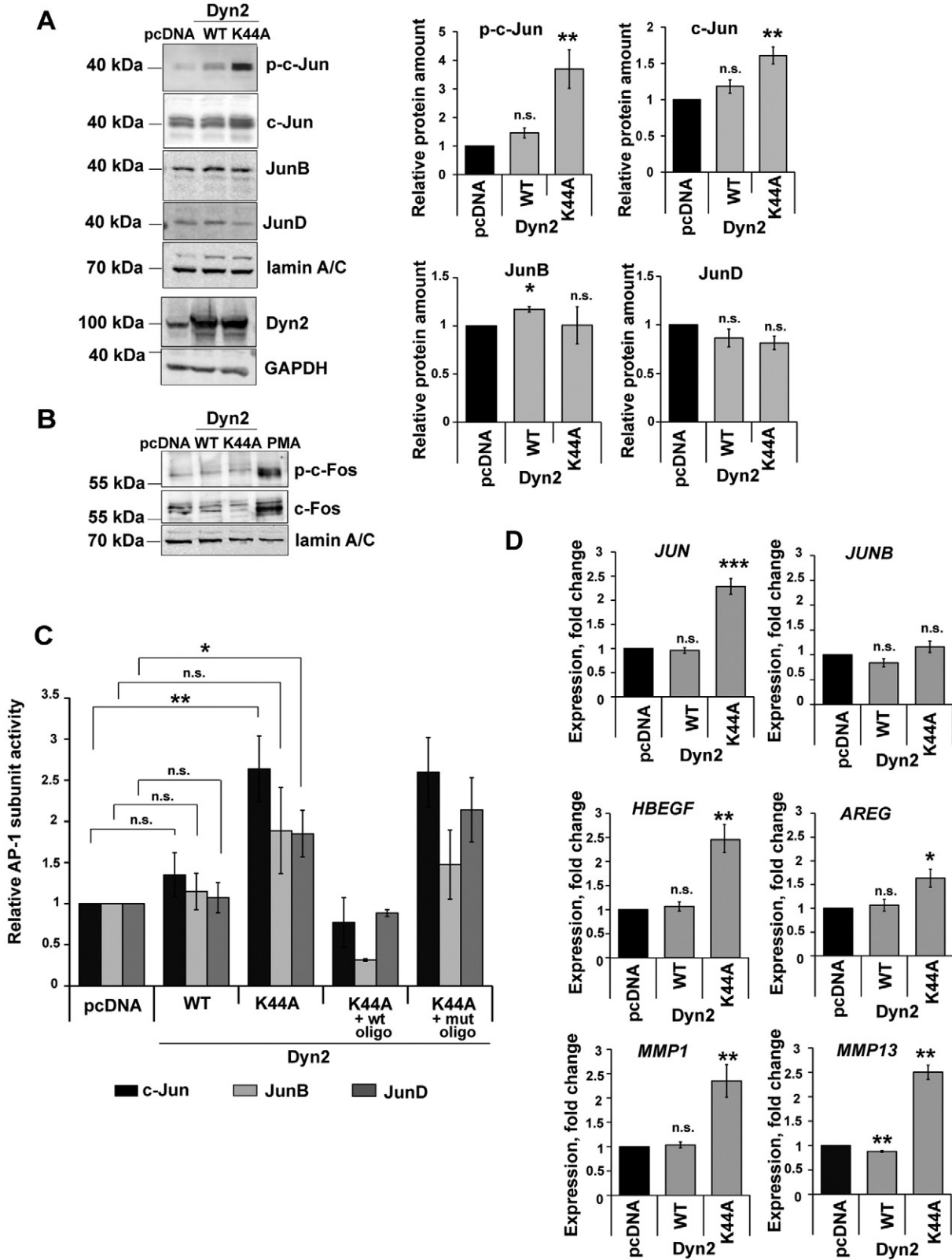
Next, we analyzed mRNA levels of four AP-1 target genes (*HBEGF*, *AREG*, *MMPI1*, *MMP13*) whose transcription is mediated by c-Jun [39–43]. Expression of the GTPase-deficient Dyn2 increased mRNA levels of all analyzed AP-1 targets. Interestingly, the observed higher expression of metalloproteinase MMP-1 upon inhibition of Dyn2 is in agreement with the reported enhanced expression of MMP-2 upon depletion of Dyn2 by siRNA in cervical carcinoma cell lines [44].

3.4. Disease-related mutants of Dyn2 activate AP-1

The K44A mutation of *DNM2* has not been found to occur in a human population, probably due to the severity of its phenotype. However, there are CMT- or CNM-related dominant-negative mutations of the human *DNM2* gene that result in an aberrant function of Dyn2 [26]. We examined the impact of four such mutations on the AP-1 activity.

We tested three CNM-related mutations: the most frequent R465W located in the middle domain of the Dyn2 protein; E650K and V625del placed in the GED and PH domains, respectively, with the latter associated with severe neonatal phenotype [45]. One tested CMT-associated mutant harbors K562E substitution in the PH domain (Fig. 3A). As shown in Fig. 3B–D, two out of four analyzed mutants, R465W and to

a lesser extent K562E, had similar effects on AP-1 as the K44A mutant. They enhanced c-Jun phosphorylation (Fig. 3B), potentiated AP-1 dependent transcription of luciferase reporter (Fig. 3C) and increased expression of AP-1 target genes (Fig. 3D). These data indicate that some CMT- and CNM-related *DNM2* mutations may alter AP-1 activity and transcription of AP-1 target genes.



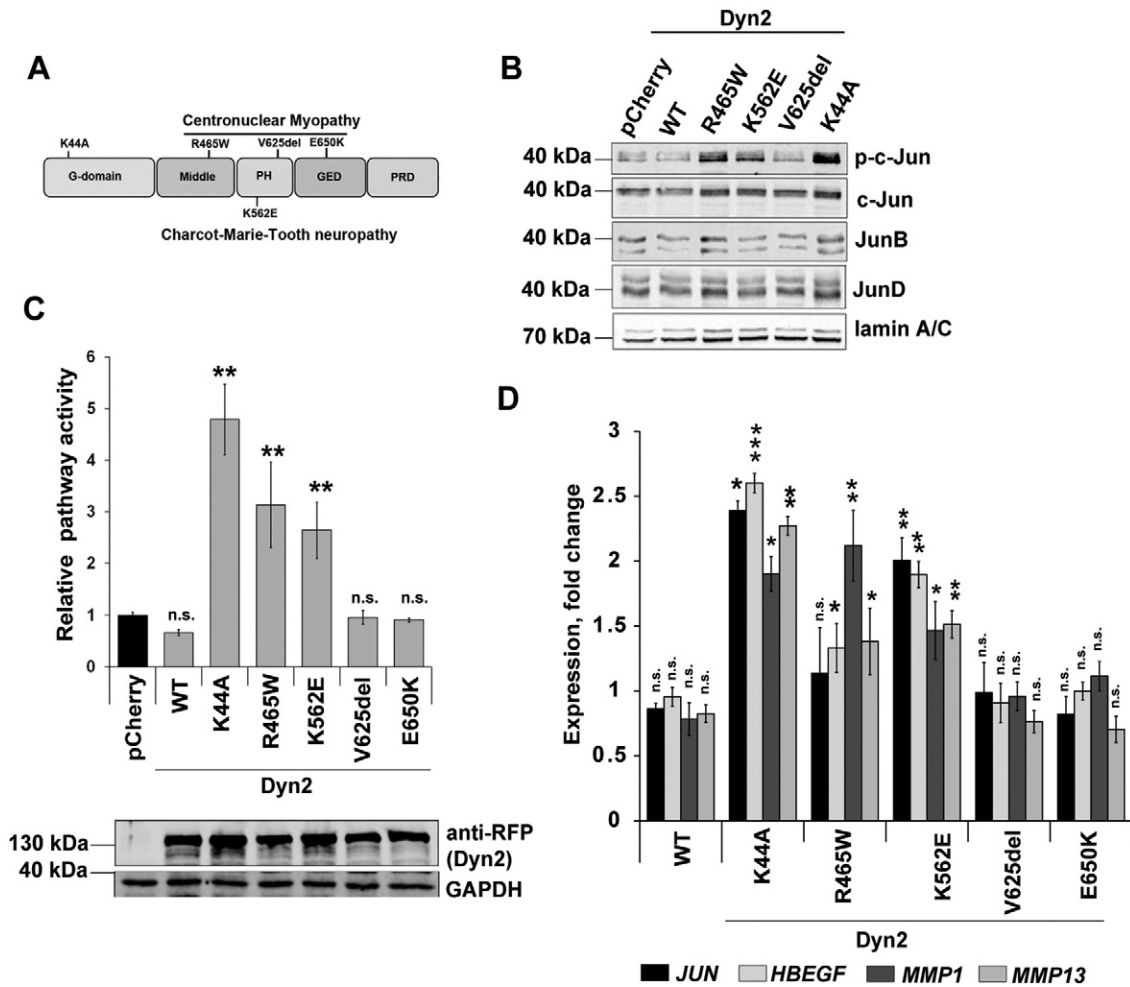


Fig. 3. *DNM2* mutations associated with human diseases may contribute to AP-1 activation. (A) Schematic representation of the Dyn2 structure. *G-domain*, GTPase domain; *Middle*, middle domain; *PH*, pleckstrin homology domain; *GED*, GTPase effector domain; *PRD*, proline rich domain. Positions of the examined point mutations and their contribution to human diseases are marked. (B) R465W and K562E mutants of Dyn2 enhance c-Jun phosphorylation on Ser63/73 (p-c-Jun). Western blot analysis of nuclear extracts from HEK293 cells transfected with the indicated constructs, with pCherry plasmid used as a vector control. Lamin A/C was used as a loading control. (C) R465W and K562E mutants of Dyn2 induce the AP-1 reporter activity. Values are mean \pm s.e.m. from 6 experiments (for K44A, R465W, K562E mutants) or 4 experiments (for V625del and E650K mutants). (D) Expression of *JUN* and AP-1 target genes upon expression of Dyn2 mutants. Values represent data averaged from 4 to 5 experiments (for WT, V625del, E650K) or 5–8 experiments (for K44A, R465W, K562E) \pm s.e.m. * $P \leq 0.05$, ** $P < 0.01$, *** $P < 0.001$ (one-sample t-test). (C–D) pCherry plasmid was used as an empty vector control for normalization (to one relative unit).

3.5. The impact of Dyn2 K44A is mediated by Src and JNKs

Next, we assessed which intracellular components of the AP-1 pathway are involved in signal transmission leading to c-Jun activation, downstream of Dyn2 K44A action. Both JNK and ERK kinases can phosphorylate Ser63/73 of the c-Jun transactivating domain, although JNKs are considerably more potent in this regard than ERK1/2 [46]. Activation of ERK1/2 occurs upon dual phosphorylation of their Thr202/Tyr204 residues, while active JNKs are phosphorylated on Thr183 and Tyr185 [47,48]. We verified that in Dyn2 K44A-expressing cells the level of phospho-ERK1/2 was unchanged (Fig. 4A), while that of phospho-

JNKs was elevated in comparison to control cells or cells overexpressing WT Dyn2 (Fig. 4B). Interestingly, Dyn2 K44A expression increased phosphorylation of both 46 and 54 kDa splicing isoforms of JNK (Fig. 4B). In line with these results, treatment of HEK293 cells with JNK inhibitor VIII diminished Dyn2 K44A-stimulated AP-1 reporter activation and c-Jun phosphorylation (Fig. 4C). The K44A mutant-induced reporter activation was also blocked by another JNK inhibitor, SP600125 (Fig. 4D). These data confirm the requirement of JNKs in Dyn2 K44A-mediated AP-1 activation.

JNK can be positively regulated by upstream tyrosine kinases such as Src [49]. We found that inhibition of Src family kinases by SU6656 or

Fig. 2. Dyn2 K44A induces c-Jun phosphorylation and expression of AP-1 target genes. (A) Expression of Dyn2 K44A increases the c-Jun protein abundance and induces its phosphorylation on Ser63/73 (p-c-Jun), without affecting amounts of JunB and JunD (Western blot on the left and quantification on the right). AP-1 subunits were analyzed in nuclear fractions, while Dyn2 expression was probed in cytosolic fractions, with lamin A/C and GAPDH as loading controls, respectively. Quantification on the right represents a mean of densitometry values from 7 (for p-c-Jun and Jun) or 3 (for JunB and JunD) independent experiments. Error bars are s.e.m. * $P \leq 0.05$, *** $P < 0.01$ (one-sample t-test). (B) c-Fos was not detected by Western blot in nuclear extracts from serum-grown HEK293 cells transfected with an empty pcDNA vector or with dynamin expressing-constructs. Nuclear extracts from PMA-stimulated cells served as a positive control for detection of total or phosphorylated c-Fos. Lamin A/C was used as loading control. (C) DNA-binding activity of p-c-Jun, JunB and JunD in nuclear extracts of HEK293 cells transfected as indicated were assayed by the TransAM® AP-1 Family kit. The binding activity in pcDNA-transfected cells was arbitrarily set to 1 relative unit. As specificity control, extracts of Dyn2 K44A-transfected cells were incubated with the competing wild-type (wt) and mutated (mut) oligonucleotides. Values are mean from 4 (p-c-Jun) or 3 (JunB and JunD) experiments \pm s.e.m. * $P \leq 0.05$, ** $P < 0.01$ (one-sample t-test). (D) Expression of AP-1 transcription factors and AP-1 target genes in cells producing WT or K44A Dyn2. Data represent mRNA levels as fold change versus control (pcDNA-transfected cells) averaged from 6 experiments, error bars are s.e.m. * $P \leq 0.05$, ** $P < 0.01$, *** $P < 0.001$ (one-sample t-test).

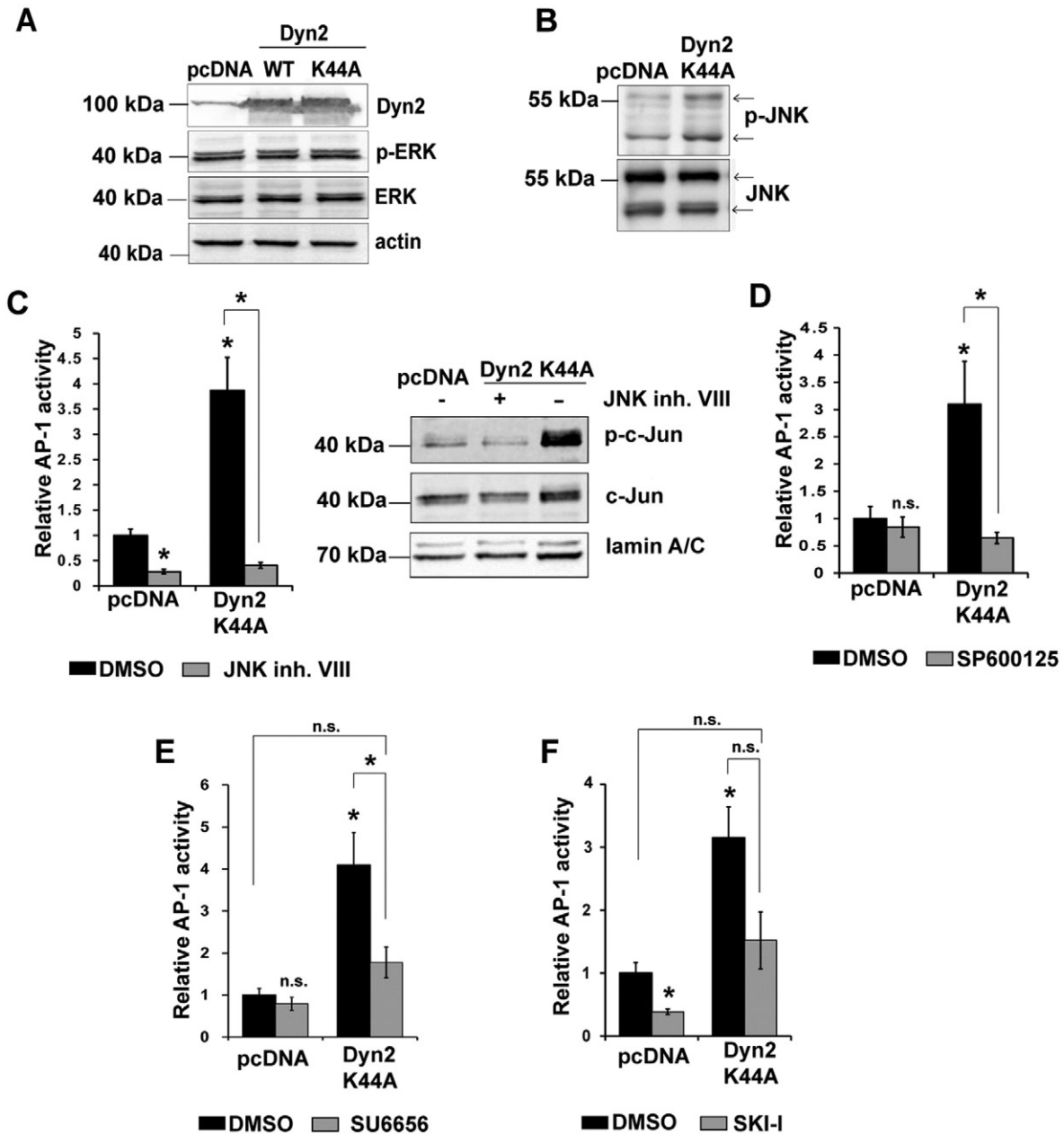


Fig. 4. Dyn2 acts at initial stages of the AP-1 pathway, upstream of Src and JNKs. (A) Dyn2 K44A does not affect ERK activation. The levels of total and active (Thr202/Tyr204 phosphorylated) ERK1/2 were analyzed by Western blotting in whole cell lysates from HEK293 cells transfected with the indicated plasmids, with actin as a loading control. (B) Dyn2 K44A increases JNK phosphorylation on Thr183 and Tyr185 without affecting its total levels (arrows indicate two isoforms of JNK, p46 and p54). (C–F) Luciferase reporter assays performed in cells transfected with plasmids as indicated. Data are normalized to the luciferase activity in pcDNA-transfected and DMSO-treated cells, which was arbitrarily set to 1 relative unit. (C, D) JNK inhibition abolishes Dyn2 K44A-mediated AP-1 induction. JNK inhibitors (10 μ M JNK inhibitor VIII in C; 50 μ M SP600125 in D) or DMSO were added to serum-grown cells 18 h before lysis. (C, right panel) Western blot analysis of Ser63/73 phosphorylation of c-Jun in nuclear extracts of JNK inhibitor VIII-treated cells. (E–F) Src kinase inhibition (2 μ M SU6656 in E; 2.5 μ M SKI-I in F) decreases both basal and Dyn2 K44A-mediated AP-1 reporter activity. Inhibitors or DMSO were applied to cells 18 h before lysis. (C–F) All values are mean \pm s.e.m. from 4 independent experiments, * P \leq 0.05 (Mann–Whitney U test).

SKI-I compounds abolished Dyn2 K44A-mediated AP-1 reporter activation (Fig. 4 E, F). This argues that, similarly to JNKs, Src kinases are required for activation of AP-1 dependent transcription downstream of Dyn2.

3.6. Dyn2 K44A stimulates AP-1 by increasing phosphorylation of several receptor tyrosine kinases (RTKs)

AP-1 activity can be controlled by extracellular stimuli, such as growth factors and their receptors. As the above described experiments were performed in the presence of serum, we hypothesized that under such conditions the constitutive stimulation of growth factor receptors

may support basal AP-1 activation, while Dyn2-mediated downregulation of these receptors would prevent the hyperactivation of AP-1. In this scenario, inhibition of endocytosis by Dyn2 K44A could lead to enhanced signaling. Such a possibility would be consistent with our data placing Dyn2 upstream of Src and JNKs in the AP-1 pathway (Fig. 4).

First, we verified the phosphorylation status of several growth factor receptors from the RTK family in serum-grown HEK293 cells (Fig. S1C). To this end, we utilized membrane arrays of antibodies recognizing 49 human RTKs in their phosphorylated, i.e. activated, forms. By comparing the relative levels of phosphorylated RTKs in lysates from serum-grown, serum-starved and serum-stimulated cells, we found that the presence of serum supported phosphorylation of several RTKs, mainly epidermal

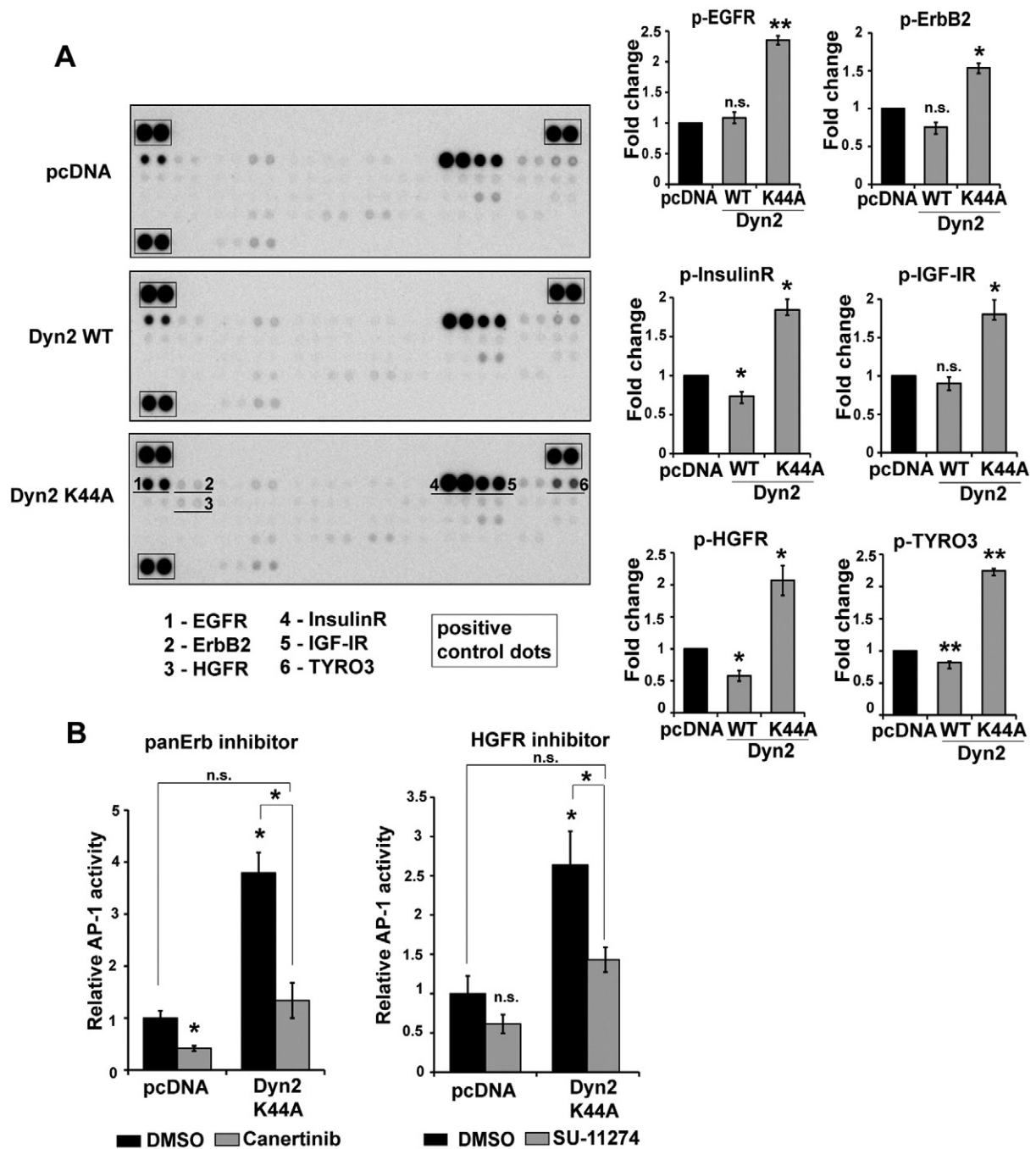
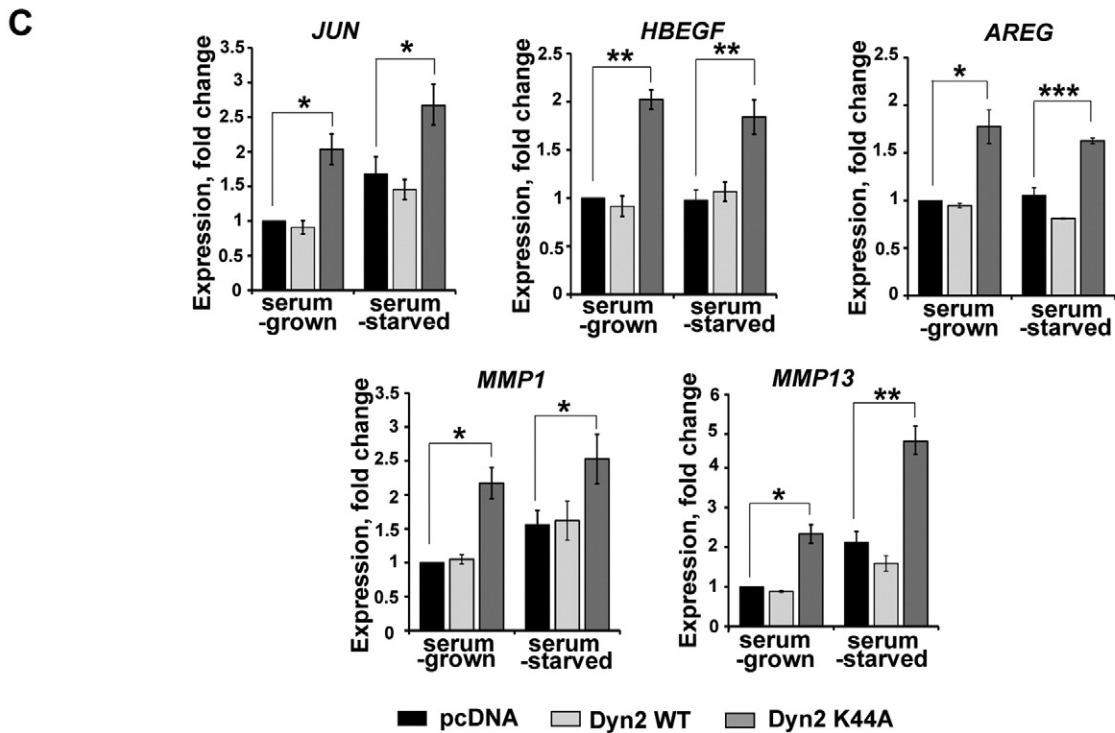
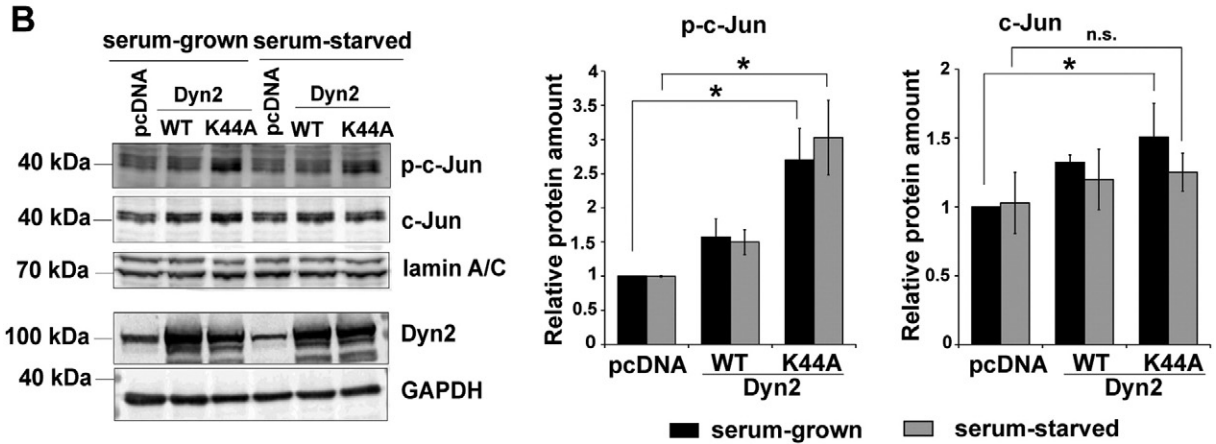
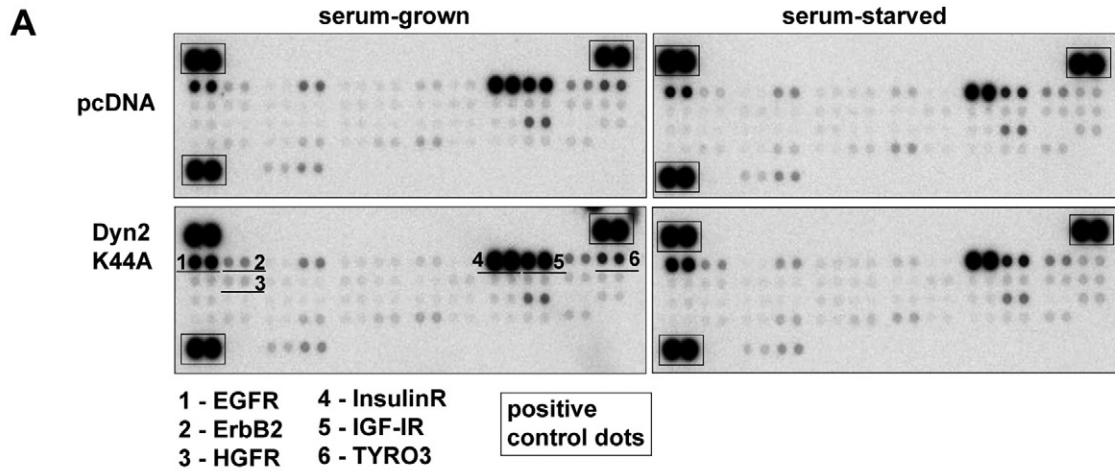


Fig. 5. Dyn2 K44A potentiates phosphorylation of RTKs. (A) Analysis of RTK phosphorylation (Human Phospho-Receptor Tyrosine Kinase Array Kit) in HEK293 cells transfected with an empty pcDNA vector or dynamin-expressing plasmids. On the left, pictures of representative array membranes with 49 human RTKs. Each pair of vertical spots represents one receptor. Paired spots in three corners are positive controls. Six receptors with increased phosphorylation upon Dyn2 K44A expression are marked and their signal quantified on the right, compared to pcDNA-transfected cells (normalized to 1 relative unit) and WT Dyn2-producing cells. Data are expressed as mean \pm s.e.m. from 3 independent experiments. * $P \leq 0.05$, ** $P < 0.01$ (one-sample t-test). (B) Dyn2 K44A-induced AP-1 reporter activity is decreased by RTK inhibitors (3 μ M Canertinib, 10 μ M SU-11274 administered to the cell medium 18 h before lysis). Data are expressed as mean \pm s.e.m. from 5 independent experiments, * $P \leq 0.05$ (Mann–Whitney U test).

growth factor receptor (EGFR), erythroblastic leukemia viral oncogene homologue 2 and 4 (ErbB2 and ErbB4), insulin-like growth factor 1 receptor (IGF1R), insulin receptor, TYRO3 and anaplastic lymphoma kinase (ALK/CD246) (Fig. S1C). The level of phosphorylation of these receptors was strongly reduced (but for EGFR, IGF1R and insulin receptor still detectable) upon serum deprivation or it was enhanced when serum-starved cells were exposed to 20% serum.

Concomitant activation of several RTKs in serum-grown cells made it unlikely that a single RTK would be responsible for AP-1 activation in

our model. Indeed, attempts to stimulate HEK293 cells with individual growth factors did not activate the AP-1 reporter (observed for EGF or hepatocyte growth factor, HGF) or stimulated it only up to 2-fold (for Gas6, a TYRO3 ligand), (data not shown). It is possible that all serum-activated RTKs function synergistically, each contributing only partially to AP-1 activation. We further verified that, when overexpressed, selected RTKs can potentially induce the AP-1 reporter activity, as shown for EGFR, platelet-derived growth factor (PDGFR) or TYRO3 (Fig. S1D). Western blot analysis of lysates from RTK-overexpressing cells



confirmed phosphorylation of Tyr residues of RTKs and Ser63/73 of c-Jun, as well as increased level of total c-Jun (Fig. S1E), similarly to the effects of Dyn2 K44A expression.

To verify our hypothesis about the impact of Dyn2 K44A on RTK signaling and AP-1 activity, we analyzed the relative levels of RTK phosphorylation in cells expressing the mutated and WT Dyn2. Indeed, we found that Dyn2 K44A enhanced phosphorylation of several RTKs (EGFR, ErbB2, insulin receptor, IGF1R, HGF receptor Met and TYRO3) (Fig. 5A). Expression of WT Dyn2 did not affect RTK phosphorylation. These data demonstrate that Dyn2 K44A induces activation of multiple receptors in serum-grown cells, which can potentially lead to stimulation of AP-1.

To confirm that RTK phosphorylation is required for Dyn2 K44A-mediated AP-1 signaling we analyzed the AP-1 reporter activity in cells treated with tyrosine kinase inhibitors targeting the receptors identified in Fig. 5A. We found that Canertinib, a pan-ErbB family inhibitor, suppressed both basal and Dyn2 K44A-induced AP-1 activity (Fig. 5B). Similarly, HGF receptor (Met) inhibitor SU-11274 decreased the AP-1 reporter under both conditions (Fig. 5B). Importantly, the action of inhibitors did not block the AP-1 reporter completely, which is consistent with a notion of multiple RTKs contributing to AP-1 activation in HEK293 cells. Unfortunately, attempts to evaluate the impact of Linsitinib (insulin receptor family inhibitor) on AP-1 reporter were inconclusive because this drug unspecifically decreased expression of a control (Renilla) luciferase. Cumulatively, these data confirmed that Dyn2 K44A-mediated AP-1 induction involves increased RTK phosphorylation which is indispensable for this effect.

3.7. The impact of Dyn2 K44A on AP-1 is largely serum-independent

Finally, we verified whether the increased RTK phosphorylation upon Dyn2 inhibition requires the presence of serum-derived growth factors which may activate the receptors. Surprisingly, in serum-starved cells, expression of Dyn2 K44A could still potentiate phosphorylation of some RTKs (Fig. 6A, right panels), although the level of induction was lower than in serum-grown cells. Consistently, we could detect increased phosphorylation of c-Jun (Fig. 6B), as well as higher expression of AP-1 target genes (Fig. 6C) which were independent of the serum presence. These data indicate that Dyn2 K44A-mediated activation of RTKs can occur in the absence of exogenous, serum-derived ligands.

4. Discussion

In this study, we demonstrate a relationship between the function of dynamin 2 and signaling via AP-1 transcription factors. We propose that under physiological conditions the activity of dynamin prevents excessive activation of AP-1 signaling. When the function of dynamin is perturbed, the JNK signaling cascade leading to AP-1 stimulation is induced. Specifically, dynamin inhibition activates the c-Jun protein, both at the transcriptional and post-translational level. The same effects, although occurring to various extents, are observed upon expression of a dominant-negative Dyn2 K44A mutant, or of certain disease-associated *DNM2* mutations.

Mechanistically, we hypothesize that the involvement of dynamin in trafficking of RTKs is a primary reason for its impact on AP-1 signaling. Under physiological conditions, dynamin-dependent endocytic trafficking of RTKs causes their downregulation from the plasma membrane, contributing to signal termination [9,50]. In turn, inhibition of dynamin-mediated internalization can lead to accumulation of

receptors on the plasma membrane which favors increased signaling [51]. This mechanism could operate also for RTK-dependent AP-1 activation. Correct endocytic trafficking of RTKs would prevent aberrant stimulation of AP-1, while their impaired internalization, due to dynamin dysfunction, would potentiate the pathway activity. To our knowledge, dynamin function has not been previously linked to AP-1 dependent transcription. However, increased JNK activity was reported upon inhibition of dynamin in pleural mesothelial cells [52] and vascular smooth muscle cells [53].

We observed that Dyn2 K44A-induced activation of RTKs is largely serum-independent. At least three different, not mutually exclusive, scenarios may be envisaged to underlie this process. One possibility is that high levels of RTKs at the plasma membrane induce their ligand-independent dimerization and activation, as reported previously [54, 55]. Secondly, some of AP-1 target genes encode growth factors (e.g. reported in this study *HBEGF* or *AREG*, encoding heparin-binding EGF or amphiregulin, respectively). Induction of their expression upon dynamin inhibition may cause an autocrine, positive feedback loop for the RTK-dependent AP-1 activation, as it has been shown for the HB-EGF-EGFR-c-Jun axis [40]. Finally, autocrine stimulation of a given RTK may induce cross-activation of another RTK, as reported for Met and EGFR [56] or for Met and IGF-1R [57].

DNM2 mutations occur in human neurological disorders. While the dominant intermediate CMT disease affects axons and myelinating Schwann cells of peripheral nerves, the CNM syndrome is linked to progressive muscular weakness with abnormal centralization of nuclei in myofibers. However, it remains unclear how the different Dyn2 mutant proteins lead to distinct diseases, affecting different cell types as specific primary targets [58]. The impact of CMT/CNM-related *DNM2* mutations on the well-established dynamin-mediated processes, i.e. clathrin-mediated endocytosis, Golgi maintenance or centrosome cohesion, have been investigated [24,59] but to our knowledge there are no reports on the effects of these mutations on signal transduction and gene expression.

Aberrant AP-1 dependent transcription has not been previously implicated in the pathophysiology of CMT or CNM but may possibly contribute to their complex and pleiotropic clinical symptoms. Our results are consistent and may explain some observations reported in the literature, mainly concerning the K562E-driven CMT. Firstly, elevated cell surface levels of ErbB2 were shown in Schwann cells expressing the K44A and CMT mutant K562E of Dyn2 [60]. Our study also shows that the K44A mutant can increase ErbB2 signaling by enhancing its phosphorylation. Importantly, ErbB2 and ErbB3 signaling is indispensable for proliferation of Schwann cells and proper myelination of peripheral nerves during development [61]. Secondly, c-Jun can be activated by ErbB2 overexpression [62] and is regarded as a negative regulator of myelination [63]. In demyelinating diseases c-Jun is upregulated in the nuclei of Schwann cells [64] which are also affected in the dynamin 2 associated intermediate form of CMT [60]. Consistently, our results identify c-Jun, among AP-1 family members, as the main target affected by Dyn2 dysfunction. Additionally, c-Jun controls expression of MMPs responsible for extracellular matrix remodeling which is also aberrant in CMT [65].

5. Conclusions

Cumulatively, here we demonstrate that inhibition of dynamin activity drives AP-1 dependent signaling, reflected by increased activation of JNK, c-Jun and enhanced target gene expression. Importantly,

Fig. 6. The impact of Dyn2 K44A on AP-1 is serum-independent. (A) Analysis of RTK phosphorylation in HEK293 cells transfected with an empty vector (pcDNA) or Dyn2 K44A-expressing plasmid, either serum-grown or serum-starved for 18 h. Representative array membranes are shown (details as in Fig. 5A). (B) Dyn2 K44A induces c-Jun phosphorylation independently of the presence of serum in cell medium. c-Jun was analyzed in nuclear fractions from serum-grown or serum-starved (18 h) cells, while Dyn2 expression was probed in cytosolic fractions, with lamin A/C and GAPDH as loading controls, respectively. Densitometry-based quantification of Western blot data (mean from 5 experiments \pm s.e.m.) is shown on the right. * $P \leq 0.05$ (one-sample t-test or independent-samples t-test). (C) Dyn2 K44A-induced expression of *JUN* and c-Jun target genes is not decreased in serum-starved cells. Data are mean \pm s.e.m. from 3 independent experiments. * $P \leq 0.05$, ** $P < 0.01$, *** $P < 0.001$ (one-sample t-test or independent-samples t-test).

disease-associated *DNM2* mutations cause similar effects on the AP-1 pathway. Thus, we propose a general principle of linking dynamin-mediated functions to transcriptional responses. This mechanism may be exploited to maintain cellular homeostasis when adapted to the needs of particular tissues or developmental stages regulated by AP-1 activity, whereas its deregulation may contribute to diseases.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.cellsig.2015.10.002>.

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