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Sprouty 2 disturbs FGFR3 degradation in thanatophoric dysplasia type II: A severe form of human achondroplasia

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Abstract

Thanatophoric dysplasia is a member of the achondroplasia family of human skeletal dysplasias, which result from FGFR3 mutations that exaggerate this receptor's inhibitory influence on chondrocyte proliferation and differentiation in the skeletal growth plate. We have previously reported that defective lysosomal degradation of activated receptor contributes to the gain-of-function of the mutant FGFR3. We now provide evidence that this disturbance is mediated by the receptor's kinase activity and involves constitutive induction and activation of Spry2. Our findings suggest that activated Spry2 may interfere with c-Cbl-mediated ubiquitination of FGFR3 by sequestering c-Cbl. They provide novel insight into the pathogenesis of this group of human skeletal dysplasias and identify a mechanism that potentially could be targeted therapeutically.

Keywords

Thanatophoric dysplasia; Achondroplasia; FGFR3; Sprouty; RTK; c-Cbl; Receptor degradation

1. Introduction

Achondroplasia (ACH) is the prototype of a group of human chondrodysplasias that range in severity from lethal thanatophoric dysplasia types I and II (TDI and TDII) to relatively mild hypochondroplasia [1,2]. These disorders result from dominantly inherited mutations of fibroblast growth factor receptor 3 (FGFR3), a transmembrane receptor tyrosine kinase (RTK) that serves as a major negative regulator of linear bone growth. The mutations are gain-of-

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function, i.e., activating mutations associated with increased tyrosine kinase activity and augmentation of FGFR3 signals [3–5]. These inhibitory signals are propagated through STAT, MAP kinase and other downstream pathways to diminish proliferation and terminal differentiation of growth plate chondrocytes [6–9].

Several mutation-specific mechanisms have been proposed to explain how different disease-related mutations lead to gain of receptor function. They include stabilization and induction of FGFR3 dimerization, which is necessary for receptor activation, by the transmembrane and cysteine substitution mutations of ACH and TDI, respectively, and kinase domain mutations of TDII that constitutively activate the receptor's intrinsic kinase activity [4,10,11]. We have recently uncovered a mechanism that may be common to ACH, TD and other activating FGFR3 mutations. Our findings suggest that mutant receptors are degraded more slowly than their wild type (WT) counterparts allowing for accumulation of actively signaling receptors and an increase in overall FGFR3 signal strength [12]. Our results point to a disturbance in c-Cbl-mediated ubiquitination of the mutant receptors as the underlying defect. Normally, receptor ubiquitination serves as a targeting signal for trafficking activated RTKs to lysosomes for degradation; consequently, defective ubiquitination slows turnover [13]. Similar disturbances have been reported in tumors due to accumulation of mitogenic RTKs [14–16].

The RTK trafficking defects in some cancers reflect somatic, loss of function mutations of c-Cbl or mutations that disrupt binding of c-Cbl to its RTK substrates, both of which impair receptor ubiquitination [14–16]. Neither of these mechanisms seems likely in the ACH group of disorders in which mutations in different regions of FGFR3 lead to the ubiquitination disturbance. More plausible is that the ubiquitination defect shared by the different FGFR3 mutations reflects the increased tyrosine kinase activity that they share [1,3]. Indeed, constitutive kinase activation has been proposed to influence maturation and trafficking of RTKs, including FGFR3 [17,18]. Also consistent with this notion is that the disturbance we previously observed in FGFR3 ubiquitination correlates with the extent of the increase in kinase activity associated with the specific mutation, i.e., moderate increase in both kinase activity and ubiquitination defect in ACH and marked increase in both kinase activity and ubiquitination defect in TD [3,12].

In this paper, we examine the possibility that accumulation of mutant FGFR3 is mediated by excessive kinase activity acting through induction and constitutive activation of Sprouty 2 (Spry2). Originally discovered in *Drosophila*, four Sprys have been identified in mammals [19,20]. They are classically induced by and antagonize MAP kinase signaling in response to several growth factors including FGF, EGF (epidermal growth factor), VEGF (vascular endothelial growth factor), PDGF (platelet-derived growth factor) and SCF (stem cell factor/kit ligand) [20–24]. Spry2 has been implicated as an FGF antagonist during lung morphogenesis and limb development and outgrowth [25–27]. Its postnatal function is less well defined, but it expressed in many adult tissues [27].

In contrast to its better known role as an inducible RTK inhibitor, Spry2 has been shown to promote RTK signaling in certain contexts through its ability to bind and sequester c-Cbl [20,28–35]. Our observations based on comparison of WT to constitutively active TDII mutant FGFR3 in cultured cells, suggest that this alternate mechanism involving Spry2 sequestration of c-Cbl may be relevant to the pathogenesis of TDII and potentially other members of the ACH group of disorders.

2. Materials and methods

2.1. Cell lines and tissues

Cell culture experiments were carried out in both COS-7 and TRex-293 (Invitrogen) cells maintained in DMEM supplemented with 10% FCS in a humidified incubator with 5% CO₂ at 37 °C. COS-7 cells stably expressing WT or TDII FGFR3-GFP were prepared by retroviral transduction under conditions designed to yield one copy of vector per infected cell as reported previously [12]. COS-7 cells stably expressing WT kinase dead (kd) or TDII kd FGFR3-GFP were prepared by lentiviral transduction using the pLenti-6 system (Invitrogen) and under conditions designed to yield 1-copy of vector per infected cell, per manufacturer's recommendation. Flp-In™ TRex-293 cell line (Invitrogen) expressing the Tet repressor and containing a single integrated Flp Recombination Target (FRT) site was used to generate tetracycline-inducible cell lines expressing GFP-tagged WT, WT kd, TDII, and TDII kd FGFR3 as follows: GFP-tagged receptors in the pcDNA5/FRT/TO cassette were co-transfected with pOG44, containing the Flp-recombinase at a 1:9 molar ratio using Lipofectamine 2000 (Invitrogen). Recombined cells were selected for hygromycin resistance and confirmed by western blot analysis.

Proximal tibial growth plate tissues were prepared from 1 week-old mice homozygous for a K644E knock-in allele corresponding to K650E TDII mutation in humans [36]. The mouse protocol was approved by the Institutional Animal Care and Use Committee. Growth plate tissues were isolated from a 17 week gestation human fetus with clinical and radiographic findings typical of TDII and a normal control human fetus 18 weeks gestation. DNA sequencing confirmed that the affected fetus had characteristic heterozygous K650E FGFR3 mutation. The human fetal tissues were obtained through the International Skeletal Dysplasia Registry under a protocol approved by Cedars-Sinai Research Institute Institutional Review Board (DK).

2.2. Reagents and antibodies

FGF1 (gift from M. Mohammadi) and FGF2 (Calbiochem) were used at final concentrations of 10 ng/ml and 25 ng/ml respectively in the presence of heparin (10 U/ml). We observed comparable responses to FGF1 and FGF2 at these doses as assayed by Erk phosphorylation. Bafilomycin (LC Laboratories, Woburn, MA) was used at 1 μM and MG132 (Calbiochem) was used at 30 μM. Anti-HA and anti-c-Cbl (C15) antibodies were obtained from Santa Cruz Biotechnology. 4G10 anti-phosphotyrosine antibody was a gift from Brian Druker, Oregon Health & Science University. Antibodies to Erk1/2 and pErk1/2 were purchased from Cell Signaling Technology, anti-GFP (Immunocytochemistry) from Abcam, anti-GFP (Western blotting) and anti-V5 from Invitrogen and anti-actin and anti-α tubulin from Sigma. Antibody to mouse Spry2 was a gift from Gerhard Christofori, University of Basel [21]; it was also purchased from Sigma (S-1444). Alexa dye conjugated secondary antibodies were purchased from Molecular Probes and HRP conjugates from Amersham.

2.3. Plasmids and transfections

The mouse FGFR3 vectors used for transient transfection were generated by excising the receptor coding region from the corresponding EGFP vectors [12] with HindIII and BamHI and subcloning into pcDNA6/V5-His (Invitrogen). The K502A mutation that abolishes the receptor's tyrosine kinase activity was introduced using QuickChange site-directed mutagenesis (Stratagene) to generate WT kinase dead (kd) FGFR3-V5-His and TDII kd FGFR3-V5-His [37]. Expression vectors for HA hSpry2 HA hSpry2 Y55F and c-Cbl and 70z-Cbl were gifts from Dafna Bar-Sagi and Brian Druker respectively, and have been previously described [12,33]. Mouse c-Cbl was amplified from pcDNA3.1(−) and cloned into dsRed2-N1 using KpnI and AgeI restriction sites following the manufacturer's recommendation (Clontech). HA-ubiquitin (HA-Ub) was a gift from Dirk Bohmann, University of Rochester

[38]. To generate the inducible TRex-293 cell lines, GFP-tagged receptors from the EGFP vector [2] were excised using HindIII and NotI and directly cloned into pcDNA5/FRT/TO. Transient transfection of COS-7 cells stably expressing FGFR3-GFP was performed using Lipofectamine 2000 (Invitrogen) or Fugene (Roche), following the manufacturer's recommended protocols. Assays were performed 30–48 h later, with or without serum starvation and following FGF1 or FGF2 stimulation. For inhibitor studies cells were serum starved 4 h prior to addition of inhibitor; inhibitor was added and 1 h later cells were stimulated with growth factor. Lysates were harvested 6 h later and receptor levels were evaluated by western blot analysis. For Spry2 induction, TRex-293 cells containing WT and TDII FGFR3-GFP were induced overnight using 2 µg/ml tetracycline. Cells were then serum starved before stimulating with 10 ng/ml FGF1 and 10 U/ml heparin for the indicated times.

2.4. Quantitative RT-PCR

TRIzol reagent for total RNA purification and the SuperScript Choice System (Invitrogen) for cDNA synthesis were used according to the manufacturers' instructions. The expression of Spry2 relative to GAPDH was determined by qRT-PCR using the ABI PRISM 7700 system (Applied Biosystems) with the following primers: sense primer for Spry2 (GGCC-TACTGTCTCGTCCCAAGAC); antisense primer for Spry2 (AGTGCTGGAGCCTAGGAGGC); sense primer for GAPDH (CATGTTTCGTCATGGGTGTGAAC); and antisense primer for GAPDH: (GTTGTCATGGATGACCTTGGC). Reactions utilized SYBR Green PCR Master Mix reagent (Applied Biosystems) according to the manufacturer's protocol. The Comparative Ct method was applied following the manufacturer's instruction to measure fold-change differences of Spry2 under each experimental condition.

2.5. Western blot and immunoprecipitation

Cells were washed twice with cold PBS and solubilized in lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.1 mM Na orthovanadate, and protease inhibitor cocktail (Roche). Human growth plate tissues were extracted on ice using a glass-teflon homogenizer. Lysis buffer extracts were clarified by centrifugation (4 °C, 14,000 rpm, 5 min) and protein measured using the Dc Protein Assay (BioRad). Samples were fractionated by SDS-PAGE, transferred to PVDF membranes and subjected to HRP-based Western blot analysis. Chemiluminescence was detected using the SuperSignal West Pico substrate following the manufacturer's instructions (Pierce). For immunoprecipitation, equivalent cell lysates were pre-cleared with 1 µg of rabbit IgG and incubated with antibodies to GFP (FGFR3-GFP), HA (HA-hSpry2, HA-Ub) or c-Cbl. Immunoprecipitates were collected with Protein G PLUS Agarose (Santa Cruz Biotechnology) and solubilized by boiling in gel sample buffer prior to Western blot analysis.

2.6. Half-life

COS-7 cells (1×10^5 cells per well, 12-well dish) were transiently transfected with Fugene following manufacturer's instructions. Forty hours post-transfection cells were washed twice in Cys/Met-free DMEM and incubated in Cys/Met-free DMEM containing 10% dialyzed serum for 30 min. Cells were then pulsed for 30 min in 150 uCi/ml Tran ³⁵S-Label (MP Biomedicals) in Cys/Met-free DMEM containing 10% dialyzed serum, rinsed three times and chased for indicated times in DMEM containing 10% serum, 100 µg/ml cold methionine and 500 µg/ml cold cysteine. Cells were rinsed in PBS, lysed in ice cold lysis buffer for 20 min at 4 °C, transferred to a 1.5 ml tube and cleared by centrifugation. Cleared lysates were immunoprecipitated overnight with 0.56 µg of anti-V5 antibody (Invitrogen) and 25 µl Protein G PLUS Agarose (Santa Cruz). Samples were run out on a 4–12% Novex Gel (Invitrogen), gel dehydrated, and ³⁵S signals were quantified using a STORM 860 Phosphorimager (GE

Healthcare). Background was subtracted and signals normalized to total protein. Numbers were plotted for 0.5 h to 7 h time point as percent total receptor versus time, and the best-fit exponential line ($R^2 \geq 96$) was plotted using EXCEL. Half-life corresponds to the time when each line crossed 50% receptor levels.

2.7. Microscopy

Partially dissected knee joints from 1 week-old WT mice and mice homozygous for the K644E FGFR3 knock-in mutation were fixed in 4% paraformaldehyde and embedded in Tissue-Tek (Sakura) for cryo-sectioning. After staining sections with anti-Spry2 antibody and Alexa 488 conjugated secondary antibody, images were captured on a Nikon Eclipse E800 epifluorescence microscope. COS-7 cells transiently expressing FGFR3 constructs were fixed with 4% paraformaldehyde, permeabilized with methanol, and immunostained using anti-GFP antibody (Abcam). Samples were imaged on a Leica TCS SP2 confocal microscope system using identical settings for image acquisition. The image files were exported as TIFF files and assembled into figures in Adobe Photoshop CS3 and Adobe Illustrator CS3.

3. Results

3.1. Kinase activity influences FGFR3 turnover

To determine if the increased tyrosine kinase activity associated with the TDII FGFR3 mutation contributes to the accumulation of mutant receptors, a separate mutation that abolishes kinase activity (K502A <K508A in humans> designated kinase dead, (kd) was introduced into WT FGFR3-V5-His and TDII FGFR3-V5-His [37]. After transient transfection, COS-7 cells were extracted and immunoblotted for receptor using a V5 antibody. The loss of kinase activity leads to a decrease in the amount of both WT and TDII receptors (Fig. 1A). This change is more pronounced for the TDII mutant receptor. This result was confirmed by a pulse-chase experiment in which COS-7 cells transiently expressing tagged WT FGFR, TDII FGFR3, or their kinase dead equivalents. Cells were pulse labeled for 30 min with ^{35}S -methionine/cysteine and chased with cold media for the designated times (Fig. 1B, C). The half-life for each receptor variant was determined from the time at which 50% protein was degraded as shown in Fig. 1C. Consistent with Fig. 1A, the TDII receptor was more stable than the WT receptor, and both kinase active receptors were more stable than their kinase dead counterparts. Parallel cultures expressing GFP-tagged receptors were immunostained for GFP using Alexa₅₆₈ secondary antibody and imaged confocally. As shown in Fig. 1D, the loss of kinase activity resulted in a slight reduction in the relative fluorescence intensity of WT FGFR3-GFP, but a substantial reduction in fluorescence intensity of TDII FGFR3-GFP.

Given our proposed model in which c-Cbl-mediated ubiquitination targets FGFR3 to lysosomes for degradation, lysosomal but not proteosomal inhibitors would be predicted to increase the relative abundance of WT and TDII FGFR3. Fig. 1E confirms this result for tagged receptors bearing active kinase. The results for kd variants was ambiguous, but a slight increase in the kd variants was observed during lysosomal inhibition.

3.2. Activated FGFR3 increases Spry2 abundance

Spry2 was considered a possible mediator of the kinase effect on FGFR3 turnover because it is typically induced by RTK signals and because of its potential to sequester c-Cbl [20,28–35]. Although FGFs are known to induce Spry2 expression, whether FGFR3 signals induce Spry2 has not been shown. Accordingly, COS-7 cells, which do not express endogenous FGFRs [39], stably expressing WT FGFR3-GFP, were treated with FGF2 and assayed for Spry2 expression by real-time RT-PCR. Fig. 2A shows a two-fold induction of Spry2 mRNA in response to FGF2 indicating FGFR3 signals can induce Spry2 transcription.

To confirm Spry2 induction at the protein level, we initially analyzed lysates from COS-7 cells stably expressing WT FGFR3-GFP as well as untransfected parental COS-7 cells; both cell types exhibited abundant Spry2 under baseline conditions (data not shown). In contrast, Spry2 was detected minimally in TRex-293 cells expressing WT FGFR3-GFP from the tet-inducible promoter as well as in parental TRex-293 cells under baseline conditions. Treatment of cells expressing WT-FGFR3-GFP with FGF1 induced Spry2 over a 4 h period (Fig. 2B). This experiment also revealed abundant Spry2 in TRex-293 cells expressing TDII FGFR3-GFP under baseline conditions that changed little with addition of ligand. Spry2 was not induced in TRex-293 cells expressing WT FGFR3-GFP lacking kinase activity. Results from four independent experiments (Fig. 2C) show that Spry2 is induced in response to FGFR3 signaling, while sustained elevated levels of Spry2 are present in cells expressing the TDII FGFR3-GFP. Additionally, a similar comparison of Spry2 in COS-7 cells stably expressing WT or TDII FGFR3-GFP consistently showed greater levels of Spry2 in cells expressing the mutant receptor (data not shown). These findings suggest that FGFR3 signals can induce Spry2 expression and that the increase in Spry2 levels is constitutive for the mutant receptor.

To document that Spry2 is expressed in postnatal growth plate chondrocytes and determine if the increase in Spry2 abundance observed in cultured cells expressing the TDII receptors is also present in vivo, proximal tibial growth plate tissues from week-old WT mice and mice homozygous for a knock-in TDII mutation were immunostained for Spry2 [36]. Fig. 3A shows staining for Spry2 in chondrocytes in the WT mouse growth plate (left panel). The right panels of Fig. 3A and B reveal an increase in Spry2 immunostaining intensity in the TDII mouse growth plate. Comparison of WT and mutant growth plates also shows a higher cell density in the TDII growth plate consistent with a reduction of hypertrophic chondrocytes typical of human TD.

Growth plate tissues for biochemical analysis were harvested from a mid-gestation human fetus with TDII and a similar age normal control. Western blot of tissue extracts fractionated by SDS-PAGE revealed a greater abundance of Spry2 in tissue from the TDII fetus when equivalent amounts of protein were loaded (Fig. 3C).

3.3. FGFR3 promotes Spry2 phosphorylation and Spry2 pY55-dependent interaction with c-Cbl

Spry2 activation involves phosphorylation of tyrosine residues, probably mediated by Src as reported previously [34,40]. To determine if FGFR3 signals can activate Spry2, cells stably expressing WT or TDII FGFR3-GFP were transfected with HA-hSpry2 and treated with FGF2 where indicated. Equivalent cell lysates (total protein) were immunoprecipitated with antibodies to HA (HA-Spry2) and then analyzed by western blot using antibodies to phosphotyrosine (pY). Fig. 4A shows that baseline phosphorylation of Spry2 is increased in response to FGF2 in cells expressing WT FGFR3, while the increased amount of Spry2 detected in cells expressing TDII FGFR3 is maximally phosphorylated independent of FGF2 treatment.

Spry2 contains seven tyrosine residues that are potentially phosphorylated by ligand or an activating mutation [40]. Y55 has been implicated as a binding site for the SH2 domain of c-Cbl when phosphorylated [34]. To test if this interaction occurs in response to FGFR3 activation, cells stably expressing WT or TDII FGFR3-GFP were transfected with WT HA-Spry2 or HA-Spry2 in which Y55 was mutated to phenylalanine (Y55F). Equivalent cell lysates were immunoprecipitated with antibody to c-Cbl and samples were analyzed by western blot with HA antibody to identify co-precipitated Spry2. Fig. 4B shows that for cells expressing WT FGFR3-GFP, WT Spry2, but not Y55F Spry2 is immunoprecipitated with c-Cbl following FGF2 stimulation. The pull-down of WT Spry2, but not Y55F Spry2, from cells expressing TDII FGFR3-GFP was independent of FGF2 treatment. These results suggest that Spry2 and

c-Cbl interact through pY55 in a ligand-dependent manner in the presence of WT FGFR3, but in a constitutive fashion for TDII FGFR3.

3.4. Spry2 acts through c-Cbl to influence FGFR3 abundance

Our observations suggest that Spry2 may exert its influence on FGFR3 stability by sequestering c-Cbl in a Spry2 pY55-dependent manner resulting in deficient receptor ubiquitination. To test this notion we asked if the reduced ubiquitination characteristic of TDII FGFR3 could be replicated for WT FGFR3 by overexpression of WT Spry2. Spry2 mutagenized to phenylalanine at Y55 (Y55F Spry2), which does not bind to c-Cbl was used as a negative control. Fig. 5A shows that when COS-7 cells stably expressing WT FGFR3-GFP were co-transfected with WT or Y55F Spry2 and HA-ubiquitin (HA-Ub), the WT Spry2 reduced FGFR3-GFP ubiquitination substantially. Y55F Spry2 also reduced FGFR3-GFP ubiquitination, but much less than WT Spry2. These findings are consistent with Spry2 sequestering c-Cbl through the Spry2 Y55:c-Cbl interaction documented in Fig. 4.

If c-Cbl sequestration by Spry2 can prolong FGFR3 half-life as we suggest, then it should be possible to overcome this effect by introducing additional c-Cbl. Accordingly, COS-7 cells were co-transfected with WT or TDII FGFR3-GFP and with increasing amounts of c-Cbl (c-Cbl-dsRed) or dominant-negative 70z-Cbl. The total amount of transfected DNA was kept constant using vector DNA. Fig. 5B shows a progressive decrease of both WT and TDII FGFR3-GFP coincident with the progressive increase of c-Cbl, whereas TDII FGFR3-GFP remained the same in the presence of increasing levels of the dominant-negative 70z-Cbl. These findings are consistent with our contention that c-Cbl is unable to target FGFR3 for degradation when it is sequestered by Spry2.

Sequestration of c-Cbl by Spry2 could enhance FGFR3 signal strength through at least two mechanisms. The first involves reduced ubiquitination as just addressed, which would prolong survival of the activated receptor and presumably augment all signaling pathways downstream of FGFR3. There is also the possibility of a direct effect on MAP kinase pathways that reflects how c-Cbl interacts with activated FGFR3. More specifically, the molecular interaction that enables c-Cbl to ubiquitinate FGFR3 is thought to involve binding of c-Cbl to a complex consisting of the docking protein Frs2 (fibroblast growth factor receptor substrate 2), which binds to the activated receptor, and the adaptor protein Grb2 (growth factor receptor-bound protein 2), to which c-Cbl binds [12,41,42]. This complex is also believed to link activated FGFRs to the MAP kinase signaling pathways that have been implicated in the pathogenesis of ACH and related disorders [8,9]. In fact, c-Cbl and SOS, which lies upstream of Ras in the MAP kinase pathways, may compete for a common binding site on Grb2 potentially creating competition between SOS-mediated MAP kinase signaling and c-Cbl-mediated FGFR3 ubiquitination [42].

This model predicts that if c-Cbl were sequestered, the putative competition between c-Cbl and SOS for Grb2 binding would be altered favoring SOS-Ras-MAP kinase signaling independent of FGFR3 ubiquitination or degradation. Distinguishing between these two possible mechanisms is beyond the scope of this paper. However, we did compare the effect of WT Spry2 to that of Y55F Spry2 on Erk phosphorylation as a readout for MAP kinase signaling in COS-7 cells stably expressing WT FGFR3. As shown in Fig. 5C WT Spry2 (HA-hSpry2), which binds to c-Cbl in a ligand-dependent fashion, enhanced Erk phosphorylation to a much greater extent than Y55F Spry2 (HA-hSpry2), which does not bind directly to c-Cbl. Taken together, these observations support the notion that Spry2 activated by FGFR3 can enhance FGFR3 signaling probably through sequestering c-Cbl.

4. Discussion

We have recently reported that FGFR3 receptors bearing mutations associated with ACH and related disorders turn over more slowly than normal, that this delay results from a disturbance of c-Cbl-mediated ubiquitination of activated FGFR3 and that the delay may contribute to the gain of receptor function associated with these disorders [12]. From in vitro experiments involving the constitutively active TDII FGFR3, we now provide evidence that the disturbance reflects the increased kinase activity of the mutant receptors and the induction and activation of Spry2. The latter events, we propose, bring into play the well-documented interaction between phosphorylated (pY55) Spry2 and c-Cbl, displacing c-Cbl from the complex thought to be responsible for FGFR3 ubiquitination [12,30,34,41,42].

Defective c-Cbl-mediated ubiquitination and degradation of oncogenic RTK proteins has been implicated in the pathogenesis of cancer, however, there are at least two important differences [15,16]. First, the somatic mutations responsible for the disturbance are clonal in cancer, i.e., restricted to the tumor, whereas they are constitutional in ACH and related disorders. Second, the mutations in tumors act in a *cis* fashion to directly impair the ubiquitin ligase activity of c-Cbl or interfere with its binding to oncogenic protein substrates. In ACH disorders, our results suggest that the effect is indirect reflecting the adverse consequences of the receptor's increased kinase activity on c-Cbl availability.

An implication from our findings is that Spry2 bound to c-Cbl would be expected to be ubiquitinated and degraded, thereby lowering its abundance, and which, in theory, could reduce its classic inhibitory effect on MAP kinase Erk signaling or alternatively its ability to sequester c-Cbl [32–34]. We did not assess Spry2 turnover, but we consistently detected higher levels of Spry2 in cells expressing TDII versus WT FGFR3 suggesting that potential loss of Spry2 due to increased turnover is more than compensated for by its increased production induced by the TDII FGFR3.

Our results suggesting that Spry2 augments MAP kinase Erk signaling through its antagonism of c-Cbl-mediated ubiquitination of FGFR3 are consistent with several reports, although primarily in the context of EGFR [20,28,29,32]. However, they differ from those described by Fong et al., who observed that Spry2 did not alter ubiquitination of FGFR1 and inhibited MAP kinase Erk signaling [30]. The reasons for the difference are not evident, although there are several possible explanations including differences in receptor and cell type and in experimental protocols. These differences may also explain why we detect deficient ubiquitination of mutant FGFR3, while Monson-Oren et al. observed an increase in ubiquitination [12,37].

A recent report from Lao et al. indicates that the regulatory unit of protein phosphatase 2 (PP2A) competes with c-Cbl for binding to Spry2 phosphorylated Y55 in the context of FGFR1 [43]. They proposed that the outcome of this competition influences both the survival of Spry2 and its regulatory influence on FGFR signaling. Our analysis of the interaction between c-Cbl and Spry2 did not examine a possible influence by PP2A. Further studies are needed to investigate its potential effect on FGFR3 signaling.

If activated Spry2 can alter FGFR3 degradation and signal output in vitro, genetic perturbation of Spry2 in animals might shed light on its physiologic significance. The knockout of Spry2 in mice has recently been reported by two groups [44,45]. Both describe poor survival and small size, but they both attribute the growth phenotype to gastrointestinal tract abnormalities. Probably more pertinent here are gain of function experiments reported by Minowada et al. [26]. Overexpression of mSpry2 in the chick limb bud dramatically shortened infected bones by blocking terminal differentiation of chondrocytes similar to that observed in human TD.

These findings are consistent with a gain of FGFR3 function in the growth plates of elongating bones as our findings would suggest.

Our experiments were not carried out in chondrocytes for technical reasons. It is conceivable that Spry2 behaves differently in chondrocytes compared with these surrogate cells or that the higher levels of vector-driven FGFR3 expression produced effects that would not be detected at physiologic levels of expression. However, results from the knock-in mouse and human TDII growth plate tissues suggest that at the least, the mutation increases the relative abundance of Spry2 in growth plate chondrocytes. Supporting this contention further is that we also detect higher amounts of Spry2 in fetal growth plate tissues from TDI compared to normal controls (unpublished observation). In vivo experiments in which lysosomal targeting of mutant FGFR3 can be evaluated under physiologic conditions, i.e., physiologic levels of FGFR3 expressed in growth plate chondrocytes in their native environment, will be needed to fully resolve these issues.

In summary, our results suggest that FGFR3 signals induce and activate Spry2, which can up modulate FGFR3 signaling through its effect on c-Cbl-mediated ubiquitination, which could slow receptor turnover. Our findings further suggest that this process is exaggerated by mutations associated with the ACH family of human chondrodysplasias that increase the intrinsic tyrosine kinase activity of the receptor. If our results are confirmed in vivo, they will help to explain the molecular pathogenesis of ACH and related disorders and identify novel targets for potential therapeutic intervention.

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References

1. Horton WA, Lunstrum GP. *Rev Endocr Metab Disord* 2002;3(4):381. [PubMed: 12424440]
2. Horton, WA.; Hecht, JT. *Connective Tissue and Its Heritable Disorders*. Royce, PM.; Steinmann, B., editors. Wiley-Liss; New York: 2002. p. 901
3. Naski MC, Wang Q, Xu J, Ornitz DM. *Nat Genet* 1996;13(2):233. [PubMed: 8640234]
4. Webster MK, Donoghue DJ. *EMBO J* 1996;15(3):520. [PubMed: 8599935]
5. Naski MC, Colvin JS, Coffin JD, Ornitz DM. *Development* 1998;125(24):4977. [PubMed: 9811582]
6. Sahni M, Ambrosetti DC, Mansukhani A, Gertner R, Levy D, Basilico C. *Genes Dev* 1999;13(11):1361. [PubMed: 10364154]
7. Minina E, Kreschel C, Naski MC, Ornitz DM, Vortkamp A. *Dev Cell* 2002;3(3):439. [PubMed: 12361605]
8. Murakami S, Balmes G, McKinney S, Zhang Z, Givol D, de Crombrughe B. *Genes Dev* 2004;18(3):290. [PubMed: 14871928]
9. Zhang R, Murakami S, Coustry F, Wang Y, de Crombrughe B. *Proc Natl Acad Sci U S A* 2006;103(2):365. [PubMed: 16387856]
10. Mohammadi M, Schlessinger J, Hubbard SR. *Cell* 1996;86:577. [PubMed: 8752212]
11. Sorokin A, Lemmon MA, Ullrich A, Schlessinger J. *J Biol Chem* 1994;269:9752. [PubMed: 8144568]
12. Cho JY, Guo C, Torello M, Lunstrum GP, Iwata T, Deng C, Horton WA. *Proc Natl Acad Sci U S A* 2004;101(2):609. [PubMed: 14699054]
13. Urbe S. *Essays Biochem* 2005;41:81. [PubMed: 16250899]
14. Thien CB, Langdon WY. *Nat Rev Mol Cell Biol* 2001;2(4):294. [PubMed: 11283727]
15. Thien CB, Walker F, Langdon WY. *Mol Cell* 2001;7(2):355. [PubMed: 11239464]

16. Peschard P, Park M. *Cancer Cell* 2003;3(6):519. [PubMed: 12842080]
17. Lievens PM, Mutinelli C, Baynes D, Liboi E. *J Biol Chem* 2004;279(41):43254. [PubMed: 15292251]
18. Schmidt-Arras DE, Bohmer A, Markova B, Choudhary C, Serve H, Bohmer FD. *Mol Cell Biol* 2005;25(9):3690. [PubMed: 15831474]
19. Hacohen N, Kramer S, Sutherland D, Hiromi Y, Krasnow MA. *Cell* 1998;92(2):253. [PubMed: 9458049]
20. Kim HJ, Bar-Sagi D. *Nat Rev Mol Cell Biol* 2004;5(6):441. [PubMed: 15173823]
21. Impagnatiello MA, Weitzer S, Gannon G, Compagni A, Cotten M, Christofori G. *J Cell Biol* 2001;152(5):1087. [PubMed: 11238463]
22. Kramer S, Okabe M, Hacohen N, Krasnow MA, Hiromi Y. *Development* 1999;126(11):2515. [PubMed: 10226010]
23. Reich A, Sapir A, Shilo B. *Development* 1999;126(18):4139. [PubMed: 10457022]
24. Zeng S, Xu Z, Lipkowitz S, Longley JB. *Blood*. 2004
25. Mailleux AA, Tefft D, Ndiaye D, Itoh N, Thiery JP, Warburton D, Bellusci S. *Mech Dev* 2001;102(1–2):81. [PubMed: 11287183]
26. Minowada G, Jarvis LA, Chi CL, Neubuser A, Sun X, Hacohen N, Krasnow MA, Martin GR. *Development* 1999;126(20):4465. [PubMed: 10498682]
27. Tefft JD, Lee M, Smith S, Leinwand M, Zhao J, Bringas P Jr, Crowe DL, Warburton D. *Curr Biol* 1999;9(4):219. [PubMed: 10074434]
28. Egan JE, Hall AB, Yatsula BA, Bar-Sagi D. *Proc Natl Acad Sci U S A* 2002;99(9):6041. [PubMed: 11983899]
29. Wong ES, Fong CW, Lim J, Yusoff P, Low BC, Langdon WY, Guy GR. *Embo J* 2002;21(18):4796. [PubMed: 12234920]
30. Fong CW, Leong HF, Wong ES, Lim J, Yusoff P, Guy GR. *J Biol Chem* 2003;278(35):33456. [PubMed: 12815057]
31. Christofori G. *Nat Cell Biol* 2003;5(5):377. [PubMed: 12724768]
32. Rubin C, Litvak V, Medvedovsky H, Zwang Y, Lev S, Yarden Y. *Curr Biol* 2003;13(4):297. [PubMed: 12593795]
33. Hall AB, Jura N, DaSilva J, Jang YJ, Gong D, Bar-Sagi D. *Curr Biol* 2003;13(4):308. [PubMed: 12593796]
34. Mason JM, Morrison DJ, Bassit B, Dimri M, Band H, Licht JD, Gross I. *Mol Biol Cell* 2004;15(5):2176. [PubMed: 15004239]
35. Mason JM, Morrison DJ, Basson MA, Licht JD. *Trends Cell Biol* 2006;16(1):45. [PubMed: 16337795]
36. Li C, Chen L, Iwata T, Kitagawa M, Fu XY, Deng CX. *Hum Mol Genet* 1999;8(1):35. [PubMed: 9887329]
37. Monsonego-Ornan E, Adar R, Rom E, Yayon A. *FEBS Lett* 2002;528(1–3):83. [PubMed: 12297284]
38. Treier M, Staszewski LM, Bohmann D. *Cell* 1994;78(5):787. [PubMed: 8087846]
39. Johnston CL, Cox HC, Gomm JJ, Coombes RC. *Biochem J* 1995;306(Pt 2):609. [PubMed: 7534069]
40. Rubin C, Zwang Y, Vaisman N, Ron D, Yarden Y. *J Biol Chem* 2005;280(10):9735. [PubMed: 15637081]
41. Hadari YR, Gotoh N, Kouhara H, Lax I, Schlessinger J. *Proc Natl Acad Sci U S A* 2001;98(15):8578. [PubMed: 11447289]
42. Wong A, Lamothe B, Lee A, Schlessinger J, Lax I, Li A. *Proc Natl Acad Sci U S A* 2002;99(10):6684. [PubMed: 11997436]
43. Lao DH, Yusoff P, Chandramouli S, Philp RJ, Fong CW, Jackson RA, Saw TY, Yu CY, Guy GR. *J Biol Chem* 2007;282(12):9117. [PubMed: 17255109]
44. Shim K, Minowada G, Coling DE, Martin GR. *Dev Cell* 2005;8(4):553. [PubMed: 15809037]
45. Taketomi T, Yoshiga D, Taniguchi K, Kobayashi T, Nonami A, Kato R, Sasaki M, Sasaki A, Ishibashi H, Moriyama M, Nakamura KI, Nishimura J, Yoshimura A. *Nat Neurosci*. 2005

Fig. 1.

Accumulation of FGFR3 is influenced by receptor tyrosine kinase activity and enhanced by lysosomal inhibition in transiently transfected cells. (A) Western blot of lysates from COS-7 cell cultures expressing WT or TDII FGFR3-V5-His with and without kinase activity using anti-V5 antibody indicates a reduced abundance of FGFR3 when kinase activity is abolished. Actin was probed as a loading control. (B) Autoradiograph resulting from a pulse-chase experiment documents a shorter half-life for kinase dead variants of WT and TDII FGFR3-V5-His. (C) Graphic representation of data represented in (B). The respective half-lives are determined from plotting normalized percent of receptor variants versus time. (D) Immunostaining of parallel cultures with anti-GFP antibody shows increased fluorescence intensity of TDII compared to WT FGFR3-GFP and reduced intensity for both receptors, especially the TDII FGFR3-GFP, when kinase activity is abolished. (E) Western blot of lysates

from COS-7 cells expressing FGFR3-GFP variants indicates that treatment with the lysosomal inhibitor Bafilomycin (Baf) but not with the proteasomal inhibitor MG132 increases relative amount of the kinase active WT and TDII FGFR3-GFP. Kinase dead mutants may increase slightly with lysosomal inhibition. Tubulin was probed as a loading control. Ct = control, V = vector only, kd = kinase dead, tub = tubulin.

Fig. 2.

Activation of FGFR3 by ligand or mutation induces expression of Spry2. (A) Real time RT-PCR shows a two-fold increase in Spry2 expression after treatment of COS-7 cells stably expressing WT FGFR3-GFP with FGF2. Results of three independent experiments are plotted showing standard deviation. (B) Representative western blots of endogenous Spry2 in lysates of TReX-293 cells expressing FGFR3-GFP variants using anti-Spry2 antibody and actin as a loading control. Spry2 is induced by FGF1 in presence of WT FGFR3-GFP, but is abundant and constitutively expressed in the presence of TDII FGFR3-GFP. Spry2 levels are low in cells expressing receptor lacking kinase activity. (C) Summarizes the results of four independent experiments. GFP-tagged WT and mutant FGFR3 expression was induced overnight using 10

μg/ml tetracycline. Cells were then made quiescent by serum starvation before stimulating with 10 ng/ml FGF1 and 10 U/ml heparin.

Fig. 3.

Spry2 is more abundant in growth plate chondrocytes from mice and humans with TDII as compared to controls. (A) Immunostaining for endogenous Spry2 in proximal tibial growth plates from 1 week-old WT (left) and TDII (K644E) (right) FGFR3 knock-in mice is more intense for the TDII growth plate. The cell density is also higher in the TDII growth plate. (B) A higher magnification of a comparable region of both growth plates outlined in (A) more clearly demonstrates differences in both staining intensity and cell density. (C) Western blot using anti-Spry2 antibody and tubulin (Tub) as a loading control demonstrates greater amount of Spry2 in growth plate tissue extracts from a human fetus with TDII (K650E) compared to a normal control.

Fig. 4.

Spry2 phosphorylation in response to FGFR3 signals leads to interaction with c-Cbl. (A) COS-7 cells stably expressing WT or TDII FGFR3-GFP were transfected with HA-hSpry2, immunoprecipitated for HA and blotted with antibody against phosphotyrosine (pY). A low level of tyrosine phosphorylation increased at 30 min following FGF2 treatment in the presence of WT FGFR3. A high level of phosphorylation of Spry2 independent of ligand stimulation was detected in cells expressing TDII FGFR3-GFP. (B) COS-7 cells stably expressing WT or TDII FGFR3-GFP were transfected with WT or Y55F HA-hSpry2, immunoprecipitated for c-Cbl and blotted for HA (HA-hSpry2). The c-Cbl immunoprecipitates contained WT, but not mutant HA-hSpry2 in the presence of both WT and TDII FGFR3-GFP. The pull-down was positive only after ligand treatment for WT FGFR3-GFP, while it was ligand-independent for TDII FGFR3-GFP. V = vector only.

Fig. 5.

Spry2 acts through c-Cbl to influence FGFR3 ubiquitination, degradation and signaling. (A) COS-7 cells stably expressing WT FGFR3-GFP were co-transfected with WT or Y55F HA-hSpry2 and HA-Ub, and equivalent lysates were immunoprecipitated for GFP (FGFR3-GFP) and blotted with antibodies to HA for HA-ubiquitin. Receptor ubiquitination was substantially reduced by WT HA-hSpry2 and much less reduced by mutant HA-hSpry2 (Y55F). (B) COS-7 cells were co-transfected with WT or TDII FGFR3-GFP and increasing amounts of c-Cbl (c-Cbl-dsRed) or dominant-negative 70z-Cbl (for TDII FGFR3-GFP) and then blotted for GFP (FGFR3-GFP) and c-Cbl. Empty-vector DNA was added to keep total amount of transfected DNA constant. Both WT and TDII FGFR3-GFP show a progressive decrease in receptor levels with increasing amounts of c-Cbl, but not with 70z-Cbl. (C) Lysates from COS-7 cells stably expressing WT-FGFR3-GFP transfected with vector, WT or Y55F HA-hSpry2 and treated or not treated with FGF2 were blotted for pErk1,2 (upper panel) and total Erk1,2 (lower panel). Addition of FGF2 for 30 min slightly increases phosphorylation of Erk1,2. Erk1,2 phosphorylation is increased substantially in the presence of WT HA-hSpry2 compared to Y55F HA-hSpry2. V = vector only, HA-Ub = HA-ubiquitin, pErk1, 2 = phosphoErk1,2.