Regulation of FOXC1 Stability and Transcriptional Activity by an Epidermal Growth Factor-activated Mitogen-activated Protein Kinase Signaling Cascade*

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Mutations in the FOXC1 transcription factor gene result in Axenfeld Rieger malformations, a disorder that affects the anterior segment of the eye, the teeth, and craniofacial structures. Individuals with this disorder possess an elevated risk for developing glaucoma. Previous work in our laboratory has indicated that FOXC1 transcriptional activity may be regulated by phosphorylation. We report here that FOXC1 is a short-lived protein (t1/2 < 30 min), and serine 272 is a critical residue in maintaining proper stability of FOXC1. Furthermore, we have demonstrated that activation of the ERK1/2 mitogen-activated protein kinase cascade through epidermal growth factor stimulation is required for maximal FOXC1 transcriptional activation and stability. Finally, we have demonstrated that FOXC1 is targeted to the ubiquitin 26 S proteasomal degradation pathway and that amino acid residues 367–553, which include the C-terminal transactivation domain of FOXC1, are essential for ubiquitin incorporation and proteolysis. These results indicate that FOXC1 protein levels and activity are tightly regulated by post-translational modifications.

Protein phosphorylation provides a rapid means of altering the function of a protein in response to changes in the cellular environment. In the case of transcription factors, phosphorylation can alter the activity of these proteins through regulation of their nuclear localization (1, 2), modulation of their protein-protein (2, 3) and/or protein-DNA (4) interactions, and by controlling their stability (5). Therefore, the phosphorylation state of a transcription factor can dictate activity and act as a molecular switch from an inactive to an active form or vice versa. The Forkhead Box transcription factor FOXC1 is an integral component for the proper formation and function of structures derived from mesoderm and neural crest lineages (6–10). In humans, mutations in the FOXC1 gene cause Axenfeld Rieger malformations, a disorder that is characterized by a spectrum of dysgeneses of the anterior segment of the eye (7, 8). The most serious consequence of this disorder is a heightenened propensity to develop glaucoma, with 50% of affected individuals developing this progressively blinding disease. In addition to the ocular findings, patients can present with a variable array of nonocular findings, including dental, craniofacial, umbilical, and cardiac anomalies. In mice, targeted deletion of both Foxc1 alleles results in neonatal lethality, with 21% of affected individuals developing this progressively blinding disease. In addition to the ocular findings, patients can present with a variable array of nonocular findings, including dental, craniofacial, umbilical, and cardiac anomalies. In mice, targeted deletion of both Foxc1 alleles results in neonatal lethality, with 21% of affected individuals developing this progressively blinding disease. In addition to the ocular findings, patients can present with a variable array of nonocular findings, including dental, craniofacial, umbilical, and cardiac anomalies. In mice, targeted deletion of both Foxc1 alleles results in neonatal lethality, with 21% of affected individuals developing this progressively blinding disease.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis—Site-directed mutagenesis of FOXC1 was performed using the QuikChange mutagenesis kit (Stratagene) as described previously (14). Mutated FOXC1 cDNAs were cloned into the pcDNA-HISMAX 4b (Invitrogen) expression vector to produce Xpress-tagged FOXC1. The mutated cDNA constructs were sequenced to confirm the fidelity of the mutagenesis reactions.

Cell Culture and Transfections—HeLa cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (FCS).2 Transient transfections with FuGENE 6 (Roche Applied Science) were performed as described previously (13). Cells were harvested 24 h post-transfection and then sonicated in a lysis buffer containing 20 mM HEPES, pH 7.9, 500 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 20% glycerol, 0.1% Triton X-100, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride and were stored at −80 °C.

FOXC1 Luciferase Reporter Assays—Transcriptional activation assays using a FOXC1-responsive luciferase reporter were performed using HeLa cells as described previously (13). Transfections were performed in triplicate, and each experiment was repeated at least three times.

Immunoblotting—Cell lysates (15 μg) were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and processed for immunoblotting as described previously (15). Xpress-tagged FOXC1 was detected with α-Xpress antibody (Invitrogen) at a dilution of 1:5000. Anti-Erk1/2 and α-phospho-Erk1/2 (Cell Signaling Technology), α-myosin heavy chain (Chemicon), α-hemagglutinin, and α-transcription factor IID (Santa Cruz Biotechnology) antibodies were all used at 1:1000. For the detection of endogenous FOXC1 proteins, 40 μg of HeLa nuclear extracts were fractionated by SDS-PAGE and proteins transferred to a nitrocellulose membrane. A rabbit polyclonal antibody raised against FOXC1 was diluted to 2 μg/ml and incubated with the membrane overnight at 4 °C. All secondary antibodies were used at a dilution of 1:5000 and incubated at room temperature for 2 h.

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2 The abbreviations used are: FCS, fetal calf serum; ERK, extracellular signal-regulated kinase; HA, hemagglutinin; Ub, ubiquitin; HA-Ub, HA-tagged ubiquitin; IMAC, immobilized metal chelate affinity chromatography; WT, wild-type; CHX, cycloheximide; MAPK, mitogen-activated protein kinase; EGF, epidermal growth factor.
FOXC1 Half-life Determination—HeLa cells were transfected with Xpress-tagged FOXC1 expression vectors. Cycloheximide (CHX) (100 \( \mu \)g/ml) was added to the cells 24 h after transfection or was added to untransfected cells for the determination of the half-life of the endogenous protein. The cells were harvested, as described for immunoblot analysis, at the appropriate time points. Protein concentrations were determined by Bradford assays, and protein expression was detected by SDS-PAGE and immunoblotting as described above. Band intensities were determined by densitometry using ImageJ (rsb.info.nih.gov/ij), and FOXC1 levels were normalized to that of ERK1/2 levels. The rate of decay was determined from linear regression analysis as described under “Experimental Procedures.”

Ubiquitin Incorporation Assay—To monitor the incorporation of ubiquitin (Ub) into FOXC1 proteins, HeLa cells were co-transfected with 2 \( \mu \)g of Xpress-tagged FOXC1 expression vector along with 2 \( \mu \)g of pMT123, an expression vector expressing influenza virus hemagglutinin (HA)-tagged Ub (generously provided by Dr. D. Bohmann, University of Rochester Medical Center), or an empty HA-tagged expression vector. Cells were harvested 24 h after transfection in lysis buffer without EDTA. Because the Xpress-tagged FOXC1 expression vectors also contained an N-terminal His\(_6\) tag, IMAC procedures were used to recover recombinant FOXC1. Six hundred micrograms of protein extract were incubated with 2 \( \mu \)g of Xpress-tagged FOXC1 expression vector along with 2 \( \mu \)Ci of \( ^{32} \)P\( \text{ATP} \) and 0.3 \( \mu \)M ATP in 1 \( \times \) kinase buffer (25 \( \mu \)M Tris, pH 7.5, 5 \( \mu \)M \( \beta \)-glycerophosphate, 2 \( \mu \)M EDTA, 1 \( \mu \)M Na\(_{2}\)VO\(_4\), and 1% Triton X-100). One hundred micrograms of HeLa cell extract were incubated with 2.5 \( \mu \)g of recombinant FOXC1 protein, 10 \( \mu \)Ci of \( ^{32} \)P\( \text{ATP} \), and 0.3 \( \mu \)M ATP in 1 \( \times \) kinase buffer (25 \( \mu \)M Tris, pH 7.5, 5 \( \mu \)M \( \beta \)-glycerophosphate, 2 \( \mu \)M dithiothreitol, 1 \( \mu \)M Na\(_2\)VO\(_4\), 50 \( \mu \)M NaF, and 10 \( \mu \)M MgCl\(_2\)) for 30 min at 30 °C. Proteins were denatured by boiling in SDS-PAGE sample buffer and resolved by SDS-PAGE. Phosphorylated proteins were detected by autoradiography and total proteins visualized by Coomassie Blue staining.

RESULTS

We focused on ERK1/2 as a potential FOXC1 kinase, because it is well established that ERK1/2 activity can regulate growth and differentiation events and because many of the developmental anomalies observed from FOXC1 mutations are suggested to result from impaired differentiation processes (6, 10, 17). Using the Motifscan search query from the Scan site data base (29), three ERK1/2 sites were predicted in FOXC1 at residues Thr-68, Ser-241, and Ser-272 (Fig. 1A). These residues were individually converted to alanines by site-directed mutagenesis. Furthermore, a missense mutation that results in a P260R substitution in FOXC1 has been detected in cervical cancers (18). The amino acid residues Thr-68, Ser-241, and Ser-272 (Fig. 1A). These residues were individually converted to alanines by site-directed mutagenesis.
Post-translational Modifications Regulate FOXC1 Stability

adjacent to Pro-260 at position 259 is a serine; this serine residue was also converted to an alanine and used in our analyses.

When the mutated FOXC1 proteins were assayed for activation of a FOXC1-responsive luciferase reporter, we found that T68A, S241A, and S259A had little effect on FOXC1 transactivation (Fig. 1B). In contrast, S272A had less than half the activity of wild-type (WT) FOXC1, suggesting that this residue can impact FOXC1 transcriptional activation. In addition, we found that S272A displayed a marked reduction in the steady state protein levels of FOXC1 compared with WT FOXC1 (Fig. 1C). This effect was reproducibly observed with five independent plasmid preparations, demonstrating that the reduced protein levels of S272A were not a result of a contaminant in the plasmid preparation, and an effect was also observed when S272A was expressed in COS-7 cells (data not shown). The levels of S241A protein were also reduced, indicating that the S241A substitution also affects FOXC1 steady state protein levels but not to the same extent as that of S272A. We co-transfected an Xpress-tagged LacZ expression vector along with WT FOXC1 or S272A and noted that the steady state levels of β-galactosidase expression were equivalent between WT FOXC1 and S272A lysates (Fig. 1D). However, the levels of FOXC1 were greatly reduced in lysates with the S272A substitution, demonstrating that impaired transfection efficiencies do not underlie the decreased levels of S272A.

Because S272A had the most profound effect on FOXC1 transactivation and on steady state protein levels, we focused our analyses on this protein. To assess whether the S272A substitution reduced the stability of FOXC1, we measured the half-lives of transfected WT FOXC1 and S272A following treatment with the protein synthesis inhibitor CHX in HeLa cells. Because the levels of S272A FOXC1 were markedly reduced compared with WT FOXC1, we utilized a 5-fold excess of S272A protein extract (100 µg) compared with levels of WT FOXC1 extract (20 µg). WT FOXC1 levels decreased upon CHX treatment (Fig. 1E) with a half-life of 65 ± 2 min (mean ± S.D.). The levels of S272A also decreased rapidly upon CHX treatment with a half-life of 47.4 ± 0.7 min (mean ± S.D.), indicating that the S272A substitution significantly reduced the stability of FOXC1 as assessed by a Student’s t test (p = 0.0065).

We next assessed whether the S272A substitution affects phosphorylation of FOXC1. We utilized a truncated FOXC1-(1–366) expression construct, which allows for a greater resolution of phosphorylated FOXC1 species (13). Several phosphorylated, immunoreactive bands (Fig. 2A, square bracket) are present in HeLa extracts transfected with WT FOXC1-(1–366); however, the slowest migrating FOXC1 band (Fig. 2A, asterisk) was not present in S272A-(1–366). These data indicate that Ser-272 is itself phosphorylated or is necessary for a FOXC1 phosphorylation event responsible for the shifted band. The remainder of the FOXC1-phosphorylated bands were still observed in the S272A-(1–366) protein, indicating that phosphorylation of FOXC1 still occurs at additional residues. The steady state protein levels of S272A-(1–366) were comparable with WT FOXC1-(1–366) levels (Fig. 2A), suggesting that amino acids 367–553 are required for proteolysis. We constructed a bacterial expression vector producing a 31-amino-acid protein corresponding to residues 259–290 of WT FOXC1 and S272A to be used as a substrate for in vitro kinase assays with HeLa cell extracts. The phosphorylation of S272A was reduced compared with WT FOXC1, indicating that S272 is itself phosphorylated or is necessary for maximal phosphorylation of this region in vitro by protein kinases present in HeLa cell extracts (Fig. 2B).

To link FOXC1 stability to specific phosphorylation events, we tested the effects of protein kinase inhibitors on FOXC1 steady state protein levels. Treatment with the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase-1 inhibitor PD98059 and LiCl greatly reduced full-length FOXC1 protein levels, indicating a role for MAPK and glycogen synthase kinase 3 signaling in the regulation of FOXC1 protein levels (Fig. 2C). HeLa cells transfected with WT or S272A were then treated with epidermal growth factor (EGF) to determine whether activation of the ERK1/2-MAPK pathway affects the levels of FOXC1 protein. As indicated in Fig. 2D, EGF administration increased levels of WT FOXC1 but had no effect on the levels of S272A FOXC1. Because the expression of both FOXC1 constructs are under...
the control of identical exogenous plasmid-derived promoters, the changes in protein levels observed are likely not to be derived from the changes observed solely in gene expression, rather they are due to an effect on protein stability that is dependent on Ser-272.

Next, we determined whether the levels and stability of endogenous FOXC1 protein respond to similar stimuli as exogenous Xpress-tagged FOXC1 protein. HeLa cells were treated with CHX, and FOXC1 protein levels were examined by immunoblot analysis. As indicated in Fig. 3A, the levels of endogenous FOXC1 begin to decline after 1 h of CHX treatment, and little FOXC1 is detected after 3 h of CHX treatment. From three independent experiments, the half-life of endogenous FOXC1 was determined to be 23 ± 6 min (Fig. 3B). The shortened half-life of the endogenous protein compared with that of the exogenous protein (Fig. 1E) reflect the substantially lower levels of endogenous FOXC1 compared with that of transfected, exogenous FOXC1. The levels of endogenous FOXC1 were increased in HeLa cells treated with CHX along with EGF but not in cells treated with EGF + PD98059, indicating that the stability of endogenous FOXC1 is regulated by EGF activation of MAPK pathways (Fig. 3C).

Activation of MAPK signaling by EGF stimulation led to a robust induction of FOXC1 transcriptional regulatory activity in cells transfected with WT FOXC1 (Fig. 4A) and then incubated in low serum conditions (0.2% FCS). This effect was diminished when cells were pre-treated with PD98059 prior to EGF administration. Activation by EGF was not observed in cells transfected with S272A, suggesting that this residue is critical for this transcriptional activation of FOXC1 in response to EGF. It should also be noted that growth in 0.2% FCS greatly reduced WT FOXC1 transcriptional regulatory activity (compare Fig. 1B with Fig. 4A). We detected little phosphorylated ERK1/2 in cells treated with 0.2% FCS, whereas ERK1/2 was robustly phosphorylated after 30 min of EGF stimulation (Fig. 4B). These results indicate that ERK1/2-MAPK signaling contributes to FOXC1 transcriptional activation in response to EGF.

Finally, we sought to determine whether FOXC1 protein stability was linked to the ubiquitin-26 S proteosome pathway. HeLa cells transfected with WT FOXC1 and S272A were incubated with the proteasomal inhibitors MG132 and proteasome inhibitor 1 (PI1). The levels of S272A were elevated in response to MG132 and PI1 treatment compared with treatment with Me2SO (Fig. 5). NH4Cl, an inhibitor of the lysosomal degradation pathway, had no effect on WT and S272A. These data suggest that the S272A substi-
tution results in a reduced stability of FOXC1 and its degradation is mediated by the 26 S proteasomal pathway. Because the 26 S proteasome recognizes and degrades proteins conjugated with polyubiquitin chains, we determined whether FOXC1 featured this covalent modification. HA-tagged ubiquitin (HA-Ub) along with Xpress-FOXC1 were co-transfected into HeLa cells, and Xpress-FOXC1 proteins were recovered by IMAC with Ni\(^{2+}\)-nitrilotriacetic acid-agarose. As indicated in Fig. 6A, numerous high molecular weight α-HA-immunoreactive bands were present in cell extracts transfected with FOXC1 and HA-Ub. When the same Ni\(^{2+}\)-nitrilotriacetic acid-agarose-treated extracts were immunoblotted with α-Xpress antibodies, these same higher molecular weight bands along with full-length FOXC1 were present on all extracts that were transfected with FOXC1. Therefore, these data indicate that FOXC1 is conjugated with polyubiquitin in vivo. To ascertain which regions of FOXC1 are required for efficient conjugation of ubiquitin to the molecule, we utilized a series of FOXC1 deletion constructs in HA-Ub conjugation assays. HA-Ub was incorporated into all FOXC1 constructs, except FOXC1-(1–366), where 187 amino acids of the C-terminal region from residue 367–553 were removed (Fig. 6B). These data along with our observations above suggest that the C-terminal region of FOXC1 is essential for efficient proteolysis.

**DISCUSSION**

In this report, we have demonstrated that FOXC1 is a short-lived transcription factor (\(t_{1/2} < 30\) min) that is degraded through the ubiquitin 26 S proteasome pathway. FOXC1 stability and activity is regulated through the ERK1/2 MAPK signaling cascades and is dependent upon Ser-272. Efficient turnover of FOXC1 requires the residues 367–553, which include the C-terminal transactivation domain. This region is either required as a substrate for attachment of ubiquitin moieties or is required for the recruitment of an E3-ubiquitin ligase. We hypothesize that phosphorylation of Ser-272 is involved in modulating protein-protein interactions that regulate FOXC1 stability (Fig. 7). In the stable state, FOXC1 is phosphorylated at Ser-272 by ERK1/2 MAPK. This phosphoserine residue may act to recruit a stabilization factor or prevent the binding of the degradation factor. This degradation factor may be a ubiquitin-E3 ligase or another protein that recruits the E3 ligase. In the unphosphorylated state, a degradation factor binds to the degron (residues 367–553), which promotes ubiquitination and degradation of FOXC1 through the 26 S proteasome pathway. The sites of ubiquitin incorporation are depicted at undetermined lysine residues on FOXC1.
Our previous work has indicated that the removal of potentially phosphorylated residues lead to a hyperactive form of FOXC1 (13). However, in this report, we have demonstrated that the removal of the phosphorylated residues at Ser-272 reduces FOXC1 stability and impairs its activity, indicating the emerging complexities of regulation of FOXC1 function by phosphorylation. Although the S272A substitution drastically reduced FOXC1 steady state protein levels, transcriptional activation by S272A was reduced only 2-fold, suggesting that the S272A protein may be less stable but more active. However, increasing the levels of S272A by 6–10 times the levels of WT FOXC1 resulted in a modest 2-fold activation by S272A, indicating that this substitution did not result in a gain of function mutation (data not shown).

The ERK1/2 MAPK pathway is integral in regulating FOXC1 activity and stability, as inhibition of ERK1/2 MAPK activity through pharmacological means or through the removal of serum growth factors can reduce FOXC1 protein steady state levels. These data indicate the possibility that FOXC1 protein stability may also be regulated throughout the cell cycle, and FOXC1 may participate in regulating cell cycle progression events necessary for the execution of differentiation programs (17, 18). Many of the clinical findings present in Axenfeld Rieger patients with FOXC1 mutations or observed in Foxc1 mutant mice are thought to arise from an impaired differentiation of neural crest mesenchyme cell populations (6, 9–11, 17, 19). Because the ERK1/2 MAPK pathway is intimately involved in cell growth and differentiation processes (20, 21), it is likely that ERK1/2 activity contributes to the regulation of FOXC1 protein steady state levels. The stability of a member of the FOX transcription factor family, FOXO1A, is also regulated by phosphorylation. An insulin and phosphatidylinositol 3-kinase-dependent signaling cascade phosphorylates FOXO1A and promotes its nuclear export (22, 23). However, the phosphorylation of FOXO1A initiates its degradation through the 26S proteasome system (23); in contrast, phosphorylation of FOXC1 by ERK1/2 MAPK signaling promotes the stability of FOXC1.

The dosage levels of FOXC1 in the eye must be exquisitely regulated, as the loss of function of a single allele is sufficient to cause anterior segment dysgeneses in humans and in mice (6–11). Moreover, chromosomal microduplications of human chromosome 6p25, the region that encompasses the FOXC1 locus, also result in a spectrum of ocular findings that include an increased susceptibility for developing glaucoma (24). A reduction in FOXC1 stability may also contribute to the Axenfeld Rieger disease pathology, as reduced steady state levels of FOXC1 protein harboring the I87M mutation have been observed (14). In light of our current findings that FOXC1 possesses a short half-life, it is likely that the I87M mutation causes human disease by destabilizing FOXC1.

The modulation of FOXC1 protein levels through post-translational modifications has ramifications for the role of FOXC1 in disease pathogenesis. Although there is complete penetrance of disease in Axenfeld Rieger patients with FOXC1 mutations, there is a high degree of variability in the phenotypes of affected individuals. In addition, only 50% of Axenfeld Rieger patients with FOXC1 mutations actually develop glaucoma. Clearly there are other mechanisms involved in the progression of the glaucoma phenotype in these individuals. One possibility is that the regulation of FOXC1 activity and its abundance through post-translational modifications augments the activity of FOXC1 protein produced from the unaffected allele, which partially compensates for the mutated allele. In this situation, these individuals will not develop glaucoma. Conversely the levels of FOXC1 protein produced from the unaffected allele may be reduced through an inactivation of the pathways involved in stabilizing FOXC1. The reduced levels of WT FOXC1 may compound the existing FOXC1 mutation, and these individuals would be more susceptible to developing glaucoma. Fluctuations in the levels of FOXC1 may also contribute to the glaucoma phenotype in individuals that do not possess FOXC1 mutations.

Glaucoma pathology is characterized by retinal ganglion cell and optic nerve degeneration that results in a progressive loss of the visual field. The elevated intraocular pressure, associated with many forms of glaucoma, has been thought to restrict the transport of neurotrophic factors to the retinal ganglion cells, and this restriction of growth factors contributes to retinal ganglion cell apoptosis, and ultimately, optic nerve degeneration (25, 26). Similarly, the removal of serum growth factors results in apoptosis of cultured retinal ganglion cells (27, 28). Because the incidence of glaucoma can be attributed to genetic, physiological, and environmental factors, it is conceivable that these factors may impinge upon the pathways that control FOXC1 protein stability. Thus the levels and activity of FOXC1 in the eye may deviate from physiologically normal levels and may underlie the glaucoma pathogenesis in a manner similar to individuals whose FOXC1 levels are modified by genetic mutations.

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