Published in final edited form as:

FASEB J. 2020 January ; 34(1): 525–539. doi:10.1096/fj.201901512R.

FIH-1 engages novel binding partners to positively influence epithelial proliferation via p63

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Abstract

Whereas much is known about the genes regulated by $\text{Np63}\alpha$ in keratinocytes, how $\text{Np63}\alpha$ is regulated is less clear. During studies with the hydroxylase, factor inhibiting hypoxia-inducible factor 1 (FIH-1), we observed increases in epidermal $\text{Np63}\alpha$ expression along with proliferative capacity in a conditional FIH-1 transgenic mouse. Conversely, loss of FIH-1 in vivo and in vitro attenuated $\text{Np63}\alpha$ expression. To elucidate the FIH-1/p63 relationship, BioID proteomics assays identified FIH-1 binding partners that had the potential to regulate p63 expression. FIH-1 interacts with two previously unknown partners, Plectin1 and STAT1 leading to the regulation of $\text{Np63}\alpha$ expression. Two known interactors of FIH-1, ASPP2 and HDAC1 were also identified. Knockdown of ASPP2 upregulated $\text{Np63}\alpha$ and reversed the decrease of $\text{Np63}\alpha$ by FIH-1 depletion. Additionally, FIH-1 regulates GADD45 α , a negative regulator of $\text{Np63}\alpha$ by interacting with HDAC1. GADD45 α knock down rescued reduction in $\text{Np63}\alpha$ by FIH-1 depletion. Collectively our data reveal that FIH-1 positively regulates $\text{Np63}\alpha$ in keratinocytes via variety of signaling partners: (i) Plectin1/STAT1; (ii) ASPP2; and (iii) HDAC1/GADD45 α signaling pathways.

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Author Contributions

Robert Lavker and Han Peng designed research and wrote the paper; Han Peng, Nihal Kaplan, Ying Dong, Sijia Wang, Wending Yang, Jong Kook Park, Junyi Wang and Elaina Fiolek performed research and analyzed data; Bethany Perez White and Navdeep S. Chandel contributed new reagents and analytic tools.

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Keywords

epidermis; limbal and corneal epithelia; GADD45 α ; ASPP2

Introduction

Central to preserving the steady-state nature of stratified epithelia are transcription factors, which control gene expression necessary for cell proliferation and differentiation. One such factor that plays a fundamental role in epithelial morphogenesis is p63, a member of the p53 family. There are six members of the p63 protein. Among them, Np63 α (1) is the main isoform in proliferating keratinocytes (2) and thought to be obligatory for proper development of epithelial structures (3, 4). High expression of p63 is correlated with elevated proliferative capacity in the epidermis, prostate and breast epithelia and supports the idea that p63 levels regulate proliferation as well as the initiation of epidermal stem cell differentiation (2, 5). Furthermore, Np63 prevents Notch signaling, which inhibits p21 expression thereby retarding epidermal differentiation (6). Paradoxically, Np63 can also: (i) synergize with Notch to induce Keratin 1 (K1) expression (7); and (ii) directly induce p57Kip2, a cyclin-dependent kinase inhibitor associated with terminal differentiation of keratinocytes (8). Collectively, these findings led to the idea that Np63 functions to promote proliferation in stem and early transit amplifying cells, and initiate programs eventuating in terminal differentiation of late transit amplifying cells (6).

Although, much is known about the genes regulated by p63 in keratinocytes and the epidermis (9–11), how p63 is regulated is less clear. A variety of E3 ligases (e.g., NEDD4, Itch), kinases (e.g., ATM, Cdk2, c-Abl), as well as p53, and stratafin/RAC1 have been implicated in the regulation of p63 protein stability (see (12) and references therein). Several miRNAs have also been implicated in the regulation of p63. For example, miR-203 directly represses the expression of p63 in keratinocytes through conserved 3'UTR binding sites, and thus suppresses proliferation (13, 14). Interestingly, several reciprocal feedback regulatory loops have been suggested to control p63. miR-130 targets p63 in keratinocytes and an autoregulatory loop has been proposed between this miRNA and Np63 (15). The relationship between p63 and the transcription factor Grainyhead-like 2 (GRHL2) is another example of a reciprocal feedback regulating p63 in keratinocytes (16).

We showed a relationship between p63 expression and FIH-1 where FIH-1 null (FIH-1^{-/-}) mice displayed a significant decrease in p63 expression in the limbal epithelium when compared with littermate controls (17). FIH-1 was originally identified as a protein that interacts with and inhibits the activity of HIF-1 α in the C-terminal transactivation domain (CAD) (18, 19) by coupling the oxidative decarboxylation of 2-oxoglutarate to the hydroxylation of HIF-1 α (20). Significantly, proteins containing the ankyrin repeat domain (ARD) such as Notch are other substrates for FIH-1 (21). Moreover, the binding affinity of FIH-1 for Notch 1 is appreciably greater than for HIF-1 α (21, 22). We demonstrated that FIH-1 can negatively regulate Notch signaling in the epidermis (23). In addition to its novel regulatory role in keratinocyte fate decisions via inactivation of Notch signaling, FIH-1 is up-regulated in the epidermis of patients with psoriasis and atopic dermatitis (23) as well as

other perturbed states (e.g., wound repair) (24). In 3-D organotypic raft cultures (3-D raft cultures) generated from primary human epidermal keratinocytes (HEKs) that ectopically expressed FIH-1, epithelial proliferation was similarly increased, with the resultant epithelial basal cells exhibiting a more proliferative phenotype compared with basal cells of control rafts (17). Collectively, FIH-1 may contribute to a “primed” or “activated” epidermis that is associated with psoriasis, atopic dermatitis and wound repair.

In the present study, we probe the roles of FIH-1 in controlling proliferation in the epidermis and the stem cell-enriched limbal epithelium (25–28) using conditional FIH-1 transgenic as well as a FIH-1 null mice. We demonstrate novel interactions between FIH-1 and STAT1, as well as between FIH-1 and plectin1, which positively affect p63 expression. We also report on an FIH-1/ASPP2/p63 α axis, which is a novel means of p63 regulation in keratinocytes. In addition, overexpression of FIH-1 attenuates GADD45 α , which is a negative regulator of Np63 (29). Such a negative regulation is achieved through a unique interaction of FIH-1 with HDAC1. Collectively, our findings indicate that FIH-1 can positively regulate p63 via four distinct signaling pathways, and this helps to explain mechanistically the association of FIH-1 overexpression with a more proliferative phenotype.

Materials and Methods

Cell culture

Primary cultures of HEKs isolated from neonatal foreskin by NU SDRC Skin Tissue Engineering core as described (23) and the limbal derived corneal epithelial cell line, hTCEpi (30), were grown in Keratinocyte SFM medium with supplements (Thermo Fisher Scientific, Massachusetts, USA) and 0.15mM CaCl₂.

Animals

A mouse with a conditional expression of the *Fih1* gene in the ROSA26 locus was produced by inserting mouse FIH-1 cDNA into a ROSA26-pCAG-stop backbone vector KI Cassette 5d by inGenious Targeting Laboratory, Inc. (Fig. S3). In this targeting vector, the expression of the *Fih1* is driven by the pCAGGS promoter and is also controlled by a stop cassette. The targeting construct was electroporated into iTL IC1 (C57BL/6) ES cells. The ROSA26-pCAG-STOP^{fl/fl}-FIH-1 C57BL/6 mice were crossed with KRT14-Cre B6CBAF1 mice purchased from The Jackson Laboratory (stock no. 004782) to obtain control and ROSA26-pCAG-FIH-1 (FIH-1 Tg) mice. The FIH-1 null mice were generated by breeding the *Fih1*-flox mouse with the *Ella*-Cre transgenic mouse (23, 24). Chemical depilation was conducted by application of a layer of Nair upon back skin for 1min. The depilatory agent and hair is removed by wiping the area with a water-moistened cloth. For BrdU labeling assay, BrdU (50 μ g BrdU/g) was injected into mice intraperitoneally. One hour post injection, tissues were processed and embedded in paraffin blocks for immunohistochemical analysis of BrdU. Animal experiments were approved by the Northwestern University Animal Care and Use Committee (NUACUC).

Constructs, Transduction and Transfection

For overexpression, a cDNA encoding FIH-1 or HDAC1-Flag was ligated between BamHI and XhoI sites of the retroviral expression plasmid LZRS (31). To conduct BioID assay, a cDNA encoding FIH-1-BirA* fusion proteins was inserted into the retroviral expression plasmid LZRS. For retroviral infections, cells were transduced with retroviral supernatants produced in Phoenix amphotropic packaging cells as previously described (23). For siRNA transfection, cells were transfected with 10 nM siRNA SMARTpools against FIH1, Plectin1, STAT1, ASPP2, GADD45 α and non-target control (GE Dharmacon, Colorado, USA) as previously described (32).

BioID proteomic assay

BioID is a novel method to screen for interacting protein partners that are in close proximity in living cells. BioID was performed as previously described (33, 34). Briefly, HEKs were transduced with a fusion of a promiscuous biotin ligase (BirA*) to FIH-1 (a bait), or an empty vector LZRS. These cells were used to generate 3-D raft cultures as previously described (23). At day 9, rafts were treated with biotin daily for 3 days. At day 12, rafts were harvested for proteins. Endogenous binding partner proteins with FIH-1 were biotinylated. These biotinylated proteins were isolated using Streptavidin beads (Santa Cruz Biotechnology, Texas, USA) under a constringent condition for identification by mass spectrometry without loss of weaker binding partners. Peptides were analyzed by LC-MS/MS using a Dionex UltiMate 3000 Rapid Separation nanoLC and a Q ExactiveTM HF Hybrid Quadrupole-OrbitrapTM Mass Spectrometer (ThermoFisher Scientific). Trap column: 150 μ m x 3 cm in-house packed with 3 μ m C18 beads. Analytical column: 75 μ m x 10.5 cm PicoChip column packed with 1.9 μ m C18 beads (New Objectives). Data were analyzed and exported using Scaffold.4.8.2 software.

Gene Ontology analysis

Functional Annotation Clustering was performed in DAVID Functional Annotation Bioinformatics Resources v6.7 and GeneGo. Genes that are regulated by wild type p63a-overexpression were exported from microarray data (GSE33495). Known binding partners of FIH-1 (substrate-trapped interactors) were exported from previous publication (PMCID: PMC4805855).

Streptavidin pulldown and co-immunoprecipitation assay

Cells were transduced with either LZRS-FIH-1-BirA* or LZRS-BirA* (control) for 48 hours. Then, cells were incubated with biotin for 24 h prior to harvest. Biotinylated proteins were pulled down using Streptavidin beads (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Co-immunoprecipitation (Co-IP) assay was performed as previously described (35). Cells were transduced with either LZRS or LZRS-FIH-1 for 48 hours. Protein lysates were incubated with 20 μ L of protein A/G PLUSAgarose beads (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) plus antibodies for FIH-1, HDAC1, STAT1, Plectin1 or ASPP2. These mixes were rotated for 2 hours at 4°C. The beads were washed three times with ice-cold phosphate-buffered saline and subjected to immunoblot analysis. NC=negative control; antibody only. WC= whole cell extract.

Western Blotting

Western blots were performed as described previously (26). The following antibodies were used: FIH-1, Grb2, tubulin, GAPDH, Notch1, GADD45 α , FLAG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), p63 α , E-cad, STAT1, pY701-STAT1, Plectin1, HDAC1, (Cell Signaling Technology, Danvers, MA, USA), ASPP2 (Abcam, Cambridge, United Kingdom), and β -catenin (Sigma-Aldrich Corp., St Louis, MO, USA).

Immunohistochemistry and immunofluorescence

Immunohistochemical (IHC), immunofluorescent (IF) or hematoxylin and eosin (H&E) staining were conducted as described previously (23). Sections were incubated with primary antibodies (1:50) overnight at 4°C. The following antibodies were used: GADD45 α (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), FIH-1, p63 α (Cell Signaling Technologies, Massachusetts, USA), K15 (Thermo Fisher Scientific, Waltham, MA, USA), Ki67, and ASPP2 (Abcam, Cambridge, United Kingdom). Sections were counterstained with DAPI. Images were taken using a Zeiss Axioplan 2 microscope system (Carl Zeiss, Oberkochen, Germany). To detect proliferation rate, BrdU staining was performed as described previously (32). Antigen retrieval of the paraffin sections was performed at 70°C in formamide retrieval solution (1X SCC in formamide) for 1hr. After blocking in PBS containing 0.01% BSA and 0.01% Tween-20, sections were incubated for 30 min with BrdU monoclonal antibody (1:10; Developmental Studies Hybridoma Bank, Iowa, USA). After washing, sections were incubated with Alexa555-linked secondary anti-mouse IgG (Thermo Fisher Scientific, Waltham, MA, USA). Images were taken using an AxioVision Z1 fluorescence microscope system (Carl Zeiss, Oberkochen, Germany). Cell counting, thickness measurement and relative fluorescence analysis were conducted by ImageJ. For superresolution structured illumination microscopy (SIM), cells were imaged with an Nikon N-SIM microscope equipped with an EM-CCD camera iXon3 DU-897E (Andor Technology, Belfast United Kingdom) and a 100 $\times$ apo 1.49-NA objective lens as described previously (32). Image reconstruction was performed with the Nikon NIS Elements software package.

Real-Time Quantitative PCR Analysis

Mouse skin epidermis was isolated from FIH-1 Tg and C57/BL6 wild type littermate control mice with or without chemical depilation. (n=3). Total RNA from epithelial sheets was purified using a miRNeasy kit (Qiagen, Valencia, CA, USA), and cDNA was prepared using a Superscript III reverse transcription kit (Invitrogen, Carlsbad, CA, USA). Real-time qPCR was performed on a Lightcycler 96 (Roche, Indianapolis, IN, USA) using SYBR green PCR kit (Roche, Indianapolis, IN, USA). Mouse *Fih1* primers were: FWD 5'-CTC GGT TGA CCC TTC AGT ATA AC; REV 5'-CAA TCC TGT GAT GGT GCC TAA. Mouse *Trp63* primers were: FWD 5'-TGT GAA ACG ATG CCC TAA CC; REV 5'-CAT GGC TGT TCC CTT CTA CTC. Mouse 18S RNA was used as the internal control. Values are fold change over wild type littermate controls.

Chromatin immunoprecipitation (ChIP) assay:

Chromatin immunoprecipitation (ChIP) assays were performed using an antibody against HDAC1 and SimpleChIP Enzymatic Chromatin IP Kit with Magnetic Beads (Cell Signaling Technology, Danvers, MA, USA) per manufacturer's instructions. DNA isolated from ChIP or 0.1% input was amplified with Roche 96 lightcycler using primers designated for GADD45 α (5'-GCTGGGGTCAAATTGCTGG-3' and 5'-GCTCGCTCGCTCCCCGGAC-3') (36). Rabbit IgG was used as isotype control.

Statistical analysis

All values are expressed as mean $\pm$ SD. The significance of the differences between 2 groups was evaluated by an unpaired Student's t test. For the differences between 3 or more groups, a 1-way ANOVA with post hoc pair-wise t-test comparisons using Bonferroni's correction for multiple comparisons was conducted. Parameters with values $P < 0.05$ were considered significant.

Results

FIH-1 levels are associated with p63 expression.

Taking an unbiased approach to interrogate the effects of FIH-1 on keratinocytes, we performed gene expression profiling on keratinocytes transduced with a FIH-1-cds versus an LZRS vector control (Table S1). We observed over 200 genes that were altered in the FIH-1 overexpressing cells (Fig. S1a). GO analysis revealed highly enriched scores for cell cycle and DNA replication (Fig. S1a). This finding correlated well with our previous observations that FIH-1 overexpression positively regulated keratinocyte proliferation in submerged and 3-D organotypic raft cultures that mimicked human epidermis (17). Since earlier work showed a relationship between p63 α and FIH-1 levels (17), we compared the genes regulated by p63 α (GSE33495) with the genes altered by FIH-1 overexpression and found a statistically significant overlap between these two groups of genes (Fig. S1b).

We have previously showed that FIH-1^{-/-} mice had a significant decrease in p63 expression in the limbal epithelium (17). Similarly, FIH-1^{-/-} adult (28 days) epidermis had markedly fewer cells that stained for p63 α compared with the wild-type littermate controls (Fig. 1a, b). In order to follow up on this FIH-1/p63 relationship, we generated a transgenic mouse whereby FIH-1 expression is activated by Cre and Cre expression is under the control of the keratin 14 promoter (FIH-1 Tg; Figs. S2a, b). The epidermis of this mouse had markedly elevated levels of FIH-1 compared to littermate controls (Figs. 1c and Fig. S2c). As predicted, the expression of p63 α was increased in the FIH-1 Tg adult (28 days) epidermis (Fig. 1d) and hair follicles (Fig. S3) compared with the wild-type littermate controls. Interestingly, there was little difference in viable epidermal thickness (VET) of the FIH-1 Tg epidermis compared with the littermate controls (Figs. 2a, b). This similarity in thickness was also reflected in equivalent amounts of cells in the "S" phase of DNA synthesis (BrdU + cells) in controls versus FIH-1 Tg mouse basal cells (Fig. 2c). It should be noted that BrdU labeling provides a snapshot of the proliferative status of a tissue at a given point in time but tells little about the proliferative capacity. Ki67 staining is reflective of the growth fraction of a given cell population (2, 5) and thus we used this marker to assess the proliferative

capacity of the FIH-1 Tg epidermis. Not surprisingly there was a marked increase in Ki67 positive cells in the FIH-1 epidermis (Fig. 2d) and combined with the increase in p63 (Fig. 2e, f) suggests that the FIH-1 Tg epidermis may be “activated” for a proliferative response. To test this, we used chemical depilation of hair, which resulted in a 40% increase in VET of the control mice (Figs. 2a, b), whereas the epidermis of the depilated FIH-1 Tg mouse had a greater than two-fold increase in VET (Figs. 2a, b). Consistent with the increases in VET, BrdU incorporation was markedly greater in the FIH-1 Tg mouse post-hair removal than in the control mice (Fig. 2c). There was a significant increase in Ki67 positive cells in the control epidermis after depilation which was not significantly different from FIH-1 Tg mouse epidermis after depilation (Fig. 2d). This was correlated with increased FIH-1 levels and p63 levels in depilated control skin (Figs. 2e, f). Collectively, these results suggest that FIH-1 primes or readies the epidermis to rapidly proliferate in response to external stimuli and there is an increase in the expression of FIH-1 after an insult to the epidermis.

Proteomics analysis identifies novel binding partners of FIH-1

To identify potential substrates of FIH-1 that regulate p63 α , we conducted BioID proximity biotinylation based proteomics analysis. We constructed a FIH-1-BirA* fusion protein and overexpressed this protein in 3-D organotypic raft cultures of HEKs as well as a limbal-derived corneal keratinocyte cell line (hTCEpi). To demonstrate whether this FIH-1-BirA* fusion protein is functional, we assessed the differentiation markers after overexpression since overexpression of FIH-1 proteins attenuates keratinocyte differentiation (17). Indeed, increased FIH-1 expression was accompanied by a reduction in differentiation markers in both HEK raft culture (e.g.K10) and hTCEpi cells (e.g.DSG3, PAI-2; Fig. S4a). Protein lysates from 3-D raft cultures with FIH-1-BirA* overexpression were subjected to mass spectrometry. We identified 247 novel FIH-1 binding partners by this unbiased BioID proteomic approach (Fig. S4b). Of these potential binding partners, we randomly picked 10 proteins and confirmed their interaction with FIH-1 using a streptavidin pulldown assay (Fig. S4c), thus validating the stringency and reproducibility of our BioID proteomic assay. We further subjected this data to three different GO analyses (Fig. S5), which indicated that the FIH-1 binding partners were enriched in known functions associated with FIH-1 such as keratinocyte differentiation, skin diseases and NOTCH signaling. Interestingly, the GO analysis also indicated the potential for FIH-1 to be involved in cell-cell junctional interactions (Fig. S5), which have not been previously considered.

FIH-1 regulates p63 α via complexing with STAT1 and plectin1

Among the potential substrates that are revealed by BioID proteomic assay, Plectin1 and STAT1 are novel interactors of FIH-1 (Fig. S5). Interestingly, both Plectin1 and STAT1 have a positive role in regulating proliferation (37–40). Although, we were not able to detect any change in the STAT1 expression between control and FIH-1 Tg mouse epidermis (Fig 3a), there was a significant enhancement in the levels of Plectin1 in FIH-1 Tg epidermis (Fig. 3b). In order to confirm FIH-1/STAT1 and FIH-1/Plectin1 association, we took advantage of SIM, and showed that both STAT1 and Plectin1 were colocalized with FIH1 (Figs 3c, d). In addition, co-immunoprecipitation assays demonstrated that FIH-1 complexed with Plectin1 and STAT1 in both hTCEpi cells and HEKs (Figs. 3e–g). To investigate the possibility that FIH-1 regulates p63 α , in part, via Plectin1 and STAT1 interactions in keratinocytes, we took

a genetic approach using siRNA constructs to knockdown Plectin1 or STAT1 and observed a decrease in p63 α levels (Fig. 4a). Knock-down of either protein was sufficient to reduce FIH-1-induced increases in p63 α expression (Fig. 4b). Similar to the FIH-1 Tg mouse epidermis, recombinant FIH-1 expression increased and FIH-1 siRNA knockdown reduced Plectin1 protein levels in HEK (Fig. 4c). These changes in FIH-1 levels did not have any effect on the expression of STAT1. Phosphorylation of STAT1 at Y701 has been shown to induce STAT1 nuclear translocation and transcriptional activity (41). We observed a slight enhancement in the pY701-STAT1 levels in HEK overexpressing FIH-1, and a reduction when FIH-1 is knocked-down (Fig. 4c). In accordance with this observation, we were able to detect increased STAT1 protein in the perinuclear region of cells knocked-down of FIH-1 (Fig. 4d), suggesting a role for FIH-1 in the cellular localization of STAT1.

FIH-1 regulates p63 α via complexing with ASPP2

ASPP2 negatively regulates p63 α and is a known substrate for FIH-1 (42, 43). To determine whether a FIH-1/ASPP2 interaction occurred in keratinocytes, we conducted streptavidin pull down as well as co-immunoprecipitation assays in HEKs (Fig. S6). Although, proteomics data did not show proximity association of FIH-1 and ASPP2, there was a clear FIH-1/ASPP2 protein-protein interaction in these cells (Fig. S6). In mice overexpressing FIH-1 (FIH-1 Tg) in the epidermis, there was a marked decrease in ASPP2 expression compared to the wild-type littermate controls, where ASPP2 was prominently expressed in epidermal basal cells (Fig. 5a). Since STAT1 can regulate and interact with ASPP2, we examined whether this regulation is dependent on STAT1 (44). We observed that knocking down STAT1 had no effect on ASPP2 levels (Fig. S7). To confirm the possibility that ASPP2 negatively regulates p63 in keratinocytes, we knocked down ASPP2 and observed an increase in p63 levels (Fig. 5b). Rescue experiments revealed that HEKs and hTCEpi cells treated with a siFIH-1 + siASPP2 returned p63 α expression towards the untreated levels (Figs. 5c, d). These results indicate that FIH-1 can positively regulate p63 α , in part, via ASPP2 repression (Fig. 5e).

FIH-1 complexes with HDAC1 and thus modulates the expression of a negative regulator of p63 α

Subjecting our gene expression profiling data to bioinformatics analysis (Table S1), we noted that FIH-1 negatively regulates GADD45 α . GADD45 α represses p63 α through p38 MAPK and p53 activation in keratinocytes (29). To confirm this novel GADD45 α regulation by FIH-1, we knocked down FIH-1 in HEKs and hTCEpi cells with an siRNA construct and observed a marked increase in GADD45 α levels (Figs. 6a, b). To investigate the possibility that FIH-1 regulates p63 α via GADD45 α in keratinocytes, we knocked down FIH-1 and GADD45 α (Figs. 6c, d). Knocking down FIH-1 in either hTCEpi or HEKs decreased p63 α expression, which could be partially rescued when both GADD45 α and FIH-1 were knocked down (Figs. 6c, d). This clearly demonstrates that FIH-1 positively regulates p63 α , in part, via inhibition of GADD45 α . A similar relationship between FIH-1, GADD45 α and p63 α expression was seen in the epidermis in vivo. Not surprisingly, there was a significant decrease in GADD45 α expression in the FIH-1 Tg epidermis versus littermate controls (Fig. 6e).

GADD45 α is not a direct substrate for FIH-1, thus to determine how FIH-1 regulates GADD45 α , we took advantage of our unbiased BioID proteomics results. Among the potential binding partners, Histone deacetylase 1 (HDAC1) is a known regulator of GADD45 α (36). Interestingly, a GST pull-down assay also showed the interaction of purified HA-FIH-1 and GST-HDAC1 proteins in solution (18). Thus we wanted to determine whether HDAC1 and FIH-1 interacted in keratinocytes. We conducted co-immunoprecipitation with antibodies against HDAC1/or FIH-1, and detected FIH-1 (Fig. 7a) or HDAC1 (Fig. 7b) in the FIH-1 transduced cultures of HEKs. Overexpression of HDAC1 reduced GADD45 α in those cells lacking FIH-1 (Fig. 7c). Moreover, using chromatin immunoprecipitation (ChIP) assay, HDAC1 binding to the chromatin regulatory regions of GADD45 α was significantly reduced in cells lacking FIH-1 (Fig. 7d), which suggests that FIH-1 regulates GADD45 α through altered binding of HDAC1 to the promoter of GADD45 α (Fig. 7e). Collectively, these observations demonstrate that FIH-1 is a positive regulator of p63 α , in part, by interfering with GADD45 α activity. Such a positive regulation of p63 α provides a mechanistic explanation for previous observations that FIH-1 was associated with a more proliferative epidermis (17).

Discussion

Much of the literature on FIH-1, has focused on the negative regulation of HIF transcriptional activity. Moreover, there has not been any in vivo gain of function studies on FIH-1, probing physiological responses. By repressing Notch1 signaling, FIH-1 attenuated epidermal differentiation and increased proliferation (17); however, the mechanisms underlying the rise in proliferation were unclear. We demonstrate that FIH-1, positively regulates proliferation, in part by increasing p63 α levels in both the epidermis and limbal epithelium. This is accomplished in three ways: (i) FIH-1 interacts with novel targets Plectin1 and STAT1, upregulating p63 α expression; (ii) FIH-1 inactivates ASPP2, resulting in increased p63 α expression; (iii) by targeting HDAC1, FIH-1 blocks GADD45 α expression, increasing p63 α expression (Fig. 7e).

FIH-1 was originally identified as a HIF-1 α interacting protein (18, 19). More recently, it has been demonstrated that the effects of FIH-1 on keratinocytes were not influenced by HIF-1 α since this molecule is inactivated by the prolyl hydroxylases (PHDs) (18, 19). Accordingly, we did not observe changes in HIF-1 α reporter activity or in levels of CA9 and VEGF, known genes downstream of HIF-1 α (45), following treatment of keratinocytes with FIH1-cds (46). Likewise, independent of HIF-1 α , we show that FIH-1 interacts with regulators of p63 α , to govern p63 α , by targeting HDAC1 upstream from GADD45 α and ASPP2, as well as interacting with STAT1 and Plectin1.

Plectin1 localizes to desmosomes and in vitro studies have shown that it can form bridges between the desmosomal protein, desmoplakin and intermediate filaments (47). Therefore, this protein is crucial to the integrity of keratinocytes and through its interaction with FIH-1 might play a role in keratinocyte proliferation (Fig 3, 4). STAT1 has been implicated in regulating proliferation in a variety of tissues (37, 39, 40) and depending on the context, has been considered both a tumor suppressor and a tumor promotor (48). However, the relationship of STAT1 with p63 was unclear until now. Our data suggests that FIH-1

regulates the nuclear localization of STAT1, which is important for transcriptional activity (Fig 4). Although it has been demonstrated that STAT1 interacts with ASPP2 as well as transcriptionally regulates ASPP2 expression (44) we were not able to detect any connection between STAT1 and ASPP2 (Suppl Fig. 7). ASPP2 inhibits Np63 expression through its ability to bind I κ B and enhance nuclear RelA/p65, which in turn mediates p63 (42). ASPP2 contains ankyrin repeat domains (ARDs), which are potential substrates for hydroxylation by FIH-1 (20, 21) and here we show their direct association (Fig. 5). Recently, ASPP2 was demonstrated to be hydroxylated at N986 and the degree of hydroxylation was dependent on FIH-1 activity (43). Interestingly, ASPP2 showed stronger binding to FIH-1 than HIF-1 α (43), in a manner similar to the binding affinity determined between Notch (another FIH-1 substrate) and FIH-1 (21, 49).

It is well-accepted that as a direct consequence of DNA damage, GADD45 α induces cell cycle arrest (50). Following UV exposure, p53-regulated DNA excision repair is controlled, in part, by GADD45 α , as evidenced by the observations that GADD45 α null mice exhibited a reduced ability for DNA repair and a higher rate of mutations, which led to a greater incidence of carcinogenesis (51). GADD45 α plays a critical role as a tumor suppressor and can inhibit p63 via p38 activation (29). Furthermore, the transcription of GADD45 α can be activated by p63 in keratinocytes thus forming a positive feedback loop (52, 53). By interacting with HDAC1, FIH-1 regulates the expression of GADD45 α to maintain p63 levels (Figs. 6, 7).

In this manner FIH-1 acts as a positive regulator of keratinocyte proliferation. Epithelial, perturbations such as wounding, result in a significant increase in FIH-1 expression compared with the resting epithelium (17, 35). This has led to the suggestion that FIH-1 may “prime” or activate the epithelial response to stress. Evidence in support of this hypothesis is the behavior of the FIH-1 Tg epidermis. The resting FIH-1 Tg epidermis does not appear morphologically distinct; however, upon perturbation, the proliferative response far exceeds that of the wild-type littermate controls. We suggest that increased p63 α levels in the FIH-1Tg epidermis may contribute to the activation or increase in proliferation. Another example of an activated epidermis is the non-lesional psoriatic skin (54–57). We have reported an increase in FIH-1 staining in the basal cells of patients with psoriasis when compared with normal individuals (23). p63 α expression has also been reported to be upregulated in psoriasis (58) and it is possible that the FIH-1/p63 α relationship described herein may reflect a possible mechanism for the increased proliferative status that is a hallmark of psoriatic lesions (59).

Previously, we demonstrated a FIH-1/LRRK1/EGFR interaction that positively affects keratinocyte migration (24). A connection between EGFR expression and p63 has been established in a pancreatic ductal adenocarcinoma cell line (60–62) with p63 being a downstream target of EGFR signaling (61). Np63 α activated EGFR transcription and 14-3-3 σ contributing to growth, migration, invasion and chemoresistance (60). While these studies were done in cell lines, the results are relevant to our present findings that FIH-1 is a positive regulator of p63 α expression in normal stratified squamous epithelia. This suggests that FIH-1 is upstream of a Np63 α /EGFR axis and further broadens the biological spectrum of role of FIH-1 in epithelial physiology.

Supplementary Material

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ACKNOWLEDGEMENTS

The NU-SDRC Morphology and Phenotyping Core facility assisted in morphologic analysis. The NU-SDRC Is supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases Grant AR057216. Proteomics services were performed by the Northwestern Proteomics Core Facility, generously supported by NCI CCSG P30 CA060553 awarded to the Robert H Lurie Comprehensive Cancer Center and the National Resource for Translational and Developmental Proteomics supported by P41 GM108569. This research is supported by National Institutes of Health Grants EY06769, EY017539 and EY019463 (to R.M.L.); National Natural Science Foundation of China grant 31300814 (to Y.D.); a Dermatology Foundation research grant and Career Development Award (to H.P.); and an Eversight research grant (to H.P.); grant K01AR072773 from the National Institute of Arthritis, Musculoskeletal and Skin Diseases (to B.P.W.).

Abbreviations

GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GSH	glutathione
H&E	hematoxylin and eosin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
EGFR	epidermal growth factor receptor
GADD45α	Growth arrest and DNA damage-45 alpha
FIH-1	Factor Inhibiting HIF-1
ASPP2	apoptosis-Stimulating Of P53 Protein 2
STAT1	Signal transducer and activator of transcription 1
HDAC1	Histone Deacetylase 1
NEDD4	Neural Precursor Cell Expressed, Developmentally Down-Regulated 4
SCC	saline-sodium citrate
VET	viable epidermal thickness
BrdU	Bromodeoxyuridine / 5-bromo-2'-deoxyuridine
BirA*	mutant biotin ligase BirA(Arg118Gly)
HEKs	human epidermal keratinocytes
hTCEpi	hTERT immortalized human corneal epithelial cell line
PHD	prolyl hydroxylases
LRRK1	LRRK1 leucine rich repeat kinase 1

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Figure 1. Knockout of FIH-1 decreases and overexpression of FIH-1 increases p63 α levels. (a) Paraffin sections of epidermis from wild-type (control) and FIH-1 null (FIH-1^{-/-}) mice at 28 (adult) days old were immunostained with antibodies against p63 α . Scale bar= 20 μ m (b) Percentage of p63 α positive cells was counted using ImageJ. Immunostaining were conducted to determine the expression of FIH-1 (c) and p63 α (d) in a FIH-1 conditional transgenic (FIH-1 Tg) mouse, whose promoter is driven by a Krt14-cre expression. Frozen sections of epidermis from WT (control) and FIH-1 Tg mice (6 weeks old) were subjected to immunostaining. K15: marking the basal epidermis (d). Relative fluorescence intensity was analyzed using ImageJ. For all experiments, n >3.

Figure 2. FIH-1 was associated with a more proliferative epidermis.

(a) Epidermis from littermate control (control) and FIH-1 Tg mice (6 weeks old) with or without depilation were subjected to H&E staining. Scale bar= 20μm (b) Thickness of epidermis was measured using ImageJ. (c, d) Epidermis from littermate control (control) and FIH-1 Tg mice (6 weeks old) with or without depilation treatment were subjected to BrdU (c) or Ki67 (d) staining. BrdU and Ki67 positive cells were counted using ImageJ. (e) Epidermis from littermate control (control) and FIH-1 Tg mice (6 weeks old) with or without depilation treatment were immunostained with p63α antibody (red). DAPI was used to stain nuclei. Scale bar= 20μm (f) *Fih1* and *Trp63* mRNA expression was determined from these mice via qRT-PCR. #: $p < 0.05$ between control and FIH-1 Tg mice. *: $p < 0.05$ between untreated and depilated epidermis. For all experiments, $n > 3$.

Figure 3. FIH-1 has novel interactions with STAT1 and Plectin1.

Epidermis from littermate control (control) and FIH-1 Tg mice (6 weeks old) were immunostained with STAT1 (a) or Plectin1 (b) antibodies (red). DAPI was used to stain nuclei. Scale bar= 50μm Colocalization of STAT1 (c) or Plectin1 (d) using SIM analysis in HEKs ectopically overexpressing FIH-1. Scale bar= 10μm Interaction of STAT1 and Plectin1 with ectopically overexpressed FIH-1 in hTCEpi and HEKs was detected by immunoprecipitation with anti-STAT1 (e), anti-Plectin1 (f) or anti-FIH-1 (g) antibodies, followed by immunoblot analysis with anti-FIH-1, anti-STAT1 or anti-Plectin1 antibodies. GAPDH was used as a loading control. NC=negative control; antibody only. WC= whole cell extract. For all experiments, n >3.

Figure 4. FIH-1 interactions with STAT1 and Plectin1 regulates p63α.

(a) hTCEpi were transfected with siSTAT1 (1,2,3,4), siPlectin1 (5,6,7,8) or scrambled control (siCTRL). Western blotting analyses were performed using antibodies against STAT1, Plectin1, p63α. Densitometry analyses were conducted from hTCEpi and HEKs using Li Cor Image Studio Lite 3.1. (b) HEKs ectopically overexpressing FIH-1 or control (LZRS) were transfected with siSTAT1, siPlectin1 or scrambled control (siCTRL). Western blotting analyses were performed using antibodies against STAT1, Plectin1, p63α. GAPDH was used as a loading control. #: $p < 0.05$ between control and ectopically expressed FIH-1. *: $p < 0.05$ between siCTRL and siPlectin1 or siSTAT1. (c) HEKs ectopically overexpressing FIH-1 or control (LZRS) were transfected with siFIH-1 or scrambled control (siCTRL). Western blotting analyses were performed using antibodies against pY701-STAT1, STAT1 and Plectin1. GAPDH was used as a loading control. #: $p < 0.05$ between control and FIH-1 Tg mice. *: $p < 0.05$ between untreated and depilated epidermis. (d) HEKs transfected with siFIH-1 or scrambled control (siCTRL) were immunostained with STAT1 (red) and FIH-1 (green).antibodies. Scale bar= 10μm. For all experiments, $n > 3$.

Figure 5. FIH-1 is a positive regulator of p63α, in part, by targeting ASPP2.

(a) Immunostaining using antibody against ASPP2 was conducted in a FIH-1 conditional transgenic (FIH-1 Tg) mouse. Epidermis from littermate control (control) and FIH-1 Tg mice (6 weeks old) were subjected to immunostaining. Relative fluorescence intensity was analyzed using ImageJ. K15: marking the basal epidermis Scale bar= 20μm (b) Western blotting showed that knockdown of ASPP2 increased p63α levels. (c, d) hTCEpi cells (c) and HEKs (d) were transfected with siFIH-1 pool, siASPP2 pool, siASPP2 pool + siFIH-1 pool, or scrambled control (siCon). Western blotting and densitometry analyses were performed. N.S.: non-significant. For all experiments, n >3.

Figure 6. FIH-1 is a positive regulator of p63α, in part, by interfering with GADD45α activity. (a, b) Western blotting showed that knockdown of FIH-1 increased GADD45α levels in both hTCEpi cells (a) and HEKs (b). (c, d) hTCEpi cells (c) and HEKs (d) were transfected with siFIH-1 pool, siGADD45α -1, siGADD45α -2, siGADD45α -1 + siFIH-1 pool, siGADD45α -2+ siFIH-1 pool, or scrambled control (siCon). Western blotting and densitometry analyses were performed. N.S.: non-significant. (e) Immunostaining were conducted to determine the expression of GADD45α in a FIH-1 conditional transgenic (FIH-1 Tg) mouse. Epidermis from littermate control (control) and FIH-1 Tg mice (6 weeks old) were subjected to immunostaining. K15: marking the basal epidermis. Scale bar= 20μm Relative fluorescence intensity was analyzed using ImageJ. For all experiments, n >3.

Figure 7. FIH-1 is a negative regulator of GADD45α, in part, by targeting HDAC1.

(a, b) Interaction of HDAC1 with ectopically overexpressed FIH-1 in HEKs was detected by immunoprecipitation with anti-HDAC1 (a) or anti-FIH-1 (b) antibodies, followed by immunoblot analysis with anti-FIH-1 (a) or anti-HDAC1 (b) antibodies, respectively. NC=negative control; antibody only. (c) HEKs were transfected with siFIH-1 pool, or scrambled control (siCTRL). These cells were transduced with FLAG-tagged HDAC1 cDNA or an empty vector (LZRS). Western blotting analyses were performed using antibodies against GADD45α, GAPDH, FLAG, HDAC1, and FIH-1. (d) HDAC1 ChIP on GADD45α regulatory region in cells KD of FIH1 (siFIH1). For all experiments, n >3. (e) Schematic representation of how FIH-1 regulates p63α expression via Plectin1/STAT1, GADD45α and ASPP2.