Immunology and Inflammation

FoxO factors are essential for maintaining organ homeostasis by acting as stress sensors in airway epithelial cells

Reviewed Preprint

Published from the original preprint after peer review and assessment by eLife.

About eLife's process

Reviewed preprint version 1
April 19, 2024 (this version)

Posted to preprint server
February 3, 2024

Sent for peer review
January 31, 2024

Karin Uliczka, Judith Bossen, Ulrich M. Zissler, Christine Fink, Xiao Niu, Mario Pieper, Ruben D. Prange, Christina Vock, Christina Wagner, Mirjam Knop, Ahmed Abdelsadik, Sören Franzenburg, Iris Bruchhaus, Michael Wegmann, Carsten B. Schmidt-Weber, Peter König, Petra Pfefferle, Holger Heine, Thomas Roeder

Division of Innate Immunity, Priority Research Area Chronic Lung diseases, Research Center Borstel – Leibniz Lung Center, Borstel, Germany • Airway Research Center North (ARCN), Member of the German Center for Lung Research (DZL) • Department of Molecular Physiology, Zoology, Kiel University, Kiel, Germany • Center of Allergy and Environment (ZAUM), Technical University Munich and Helmholtz Center Munich, German Research Center for Environmental Health, Munich, Germany • CPC-M, Member of the German Center for Lung Research (DZL) • Anatomical Institute, Lübeck University, Lübeck, Germany • Division of Experimental Pneumology, Priority Research Area Chronic Lung diseases, Research Center Borstel – Leibniz Lung Center, Borstel, Germany • Division of Invertebrate Models, Priority Research Area Chronic Lung diseases, Research Center Borstel – Leibniz Lung Center, Borstel, Germany • Zoology Department, Faculty of Science, Aswan University, 81528, Aswan, Egypt • IKMB, Kiel University, Kiel, Germany • Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany • Division of Lung Immunology, Priority Research Area Chronic Lung diseases, Research Center Borstel – Leibniz Lung Center, Borstel, Germany • Medical Faculty, University Medical Center Giessen and Marburg, Philipps-Universität Marburg, Marburg, Germany • UGMLC, Member of the German Center for Lung Research (DZL)

Abstract

Airway epithelia have the challenging task of maintaining functional and structural homeostasis, even when exposed to various stress factors. Transcription factors of the FoxO family can fulfill this complex task, as they act as integration hubs that translate extrinsic and intrinsic information into a physiologically appropriate response. We could show that FoxO factors in *Drosophila*, mouse, and human airway epithelial cells (AECs) respond to stressors like hypoxia, temperature, or oxidative stress by nuclear translocation. A complex activation pattern is revealed in human cell culture systems, which differs between individual hFOXO factors and cell types. Studies with *Drosophila* showed that hypoxia was the only stressor that induced a dfoxo-dependent, local immune response activation. Since *Drosophila* has only one ortholog of FoxO, it was possible to show that the absence of dfoxo in the airways strongly increases the stress sensitivity of the airways. This stress sensitivity finds its counterpart in mouse models of chronic and acute asthma, with reduced mFoxO expression in the lung, particularly mFoxO1 and mFoxO3A. Finally, it is also reflected in asthma patients who show reduced hFOXO transcripts in their sputum samples. We conclude that active FoxO signaling in AECs is necessary to respond appropriately to stressors. Impaired FoxO signaling limits this ability and thus promotes disease development.

eLife assessment

This **important** study investigates, from Drosophila to mammals, the role of the Forkhead box O (FoxO) transcription factors in airway epithelial cells' response to stressors including hypoxia, temperature variations, and oxidative stress. The findings suggest a conserved role of FoxO in maintaining airway homeostasis across species. However, limitations in the specificity and concerns with the loss-of-function experiments render the evidence presented **incomplete**. Nonetheless, this study highlights FoxO's potential relevance in respiratory diseases like asthma and offers insights into potential therapeutic targets for conditions affecting airway health.

1 INTRODUCTION

Airway epithelial cells (AECs) form the barrier between the respiratory organs and the environment. Therefore, AECs are permanently exposed to physical stressors, pathogens, environmental pollutants, and allergens. A significant task of AECs is to orchestrate the lung's responses to these environmental influences, thereby maintaining airway structural integrity and immune homeostasis (Hewitt & Lloyd, 2021; Weisberg *et al*, 2021; Whitsett & Alenghat, 2015). Impairment of these functions is associated with developing chronic lung diseases such as asthma, chronic obstructive pulmonary disease (COPD), or pulmonary fibrosis, supporting the hypothesis that these diseases are primarily epithelial (Celebi Sozener *et al*, 2020; Heijink *et al*, 2020; Hellings & Steelant, 2020; Lambrecht & Hammad, 2012). This interpretation is further supported by the fact that carriers of risk alleles for these diseases are particularly prone to develop deregulated epithelial immune functions (Hellings & Steelant, 2020; Pividori *et al*, 2019; Raby *et al*, 2023) and inappropriate repair responses (Burgoyne *et al*, 2021; Chung & Adcock, 2008). Significant disease hallmarks comprise airway remodeling and prolonged, persistent inflammatory reactions (McAlinden *et al*, 2019; Proud & Leigh, 2011; Spann *et al*, 2014; Zepp & Morrisey, 2019).

This central property of AECs to respond appropriately to a wide variety of intrinsic and extrinsic signals requires signaling systems that react to these signals and connect their activation with an appropriate cellular response. The transcription factors of the FoxO family precisely fulfill these requirements (Gui & Burgering, 2022; Orea-Soufi *et al*, 2022). They are critically involved in various cellular processes, including apoptosis, cell survival, oxidative stress responses, and energy metabolism (Graves & Milovanova, 2019). While the fruit fly *Drosophila melanogaster* has only one FoxO gene (*dfoxo*) (Junger *et al*, 2003), mammals have four FoxO factors (in humans: hFOXO1, hFOXO3, hFOXO4, and hFOXO6). In all these organisms, these factors act as integration hubs for different cellular signaling systems, such as the insulin and JNK pathways, responding to various signals (Cheng, 2019; Greer & Brunet, 2005; van der Vos & Coffer, 2011; Wagner *et al*, 2021).

The interest in FoxO factors is largely based on research efforts in two different scientific fields: aging and research on a broad spectrum of chronic diseases. In aging studies, increased FoxO activity usually correlates with a prolonged lifespan. This correlation initially found in models such as *C. elegans* and *Drosophila* (Giannakou *et al*, 2007; Sun *et al*, 2017), is further supported by the fact that *hFOXO3A* is the only human longevity gene identified to date (Donlon *et al*, 2022; Flachsbart *et al*, 2009). On the other hand, deregulation of hFOXO activities has been associated with various chronic human diseases (Manolopoulos *et al*, 2010; Orea-Soufi *et al*., 2022; van der Horst & Burgering, 2007; Xing *et al*, 2018). This causal relationship between altered hFOXO activity and disease pathogenesis has also been demonstrated for chronic lung diseases. Thus, a

deficiency of the transcription factor hFOXO1 contributes to developing pulmonary hypertension (Savai *et al*, 2014; Selle *et al*, 2022). Deficiencies in *hFOXO3*, on the other hand, correlate with COPD and idiopathic pulmonary fibrosis (IPF) (Al-Tamari *et al*, 2018; Hwang *et al*, 2011). Moreover, alterations in the expression of *hFOXO* factors were associated with various cancer entities including lung cancer (Hornsveld *et al*, 2018; Jiramongkol & Lam, 2020; Mikse *et al*, 2010). In human respiratory epithelial cells, increased activation of the hFOXO signaling pathway directly controls an epithelial immune response, a mechanism first demonstrated in *Drosophila* (Becker *et al*, 2010; Seiler *et al*, 2013). Consistently, rhinoviral infection activates hFOXO3 in airway epithelia and helps to orchestrate antiviral responses (Gimenes-Junior *et al*, 2019). Recently, general aspects of FoxO biology in epithelial tissues were elucidated in *Drosophila* (Fink *et al*, 2016; Wagner *et al*., 2021).

To gain deeper insight into the importance of FoxO-mediated signaling in AECs, we performed analyses using *Drosophila*, mice, and humans. Our studies revealed the highly evolutionarily conserved activation of FoxO factors by various stressors in AECs. In human cells, nuclear translocation of hFOXO factors differed depending on the cell- and hFOXO type. Furthermore, *dfoxo* deficiency was associated with reduced stress tolerance in *Drosophila*. Indeed, the role of FoxO factors as hubs for the integration of stress signals was also evident in asthma mouse models, in which the amount of *mFoxo* transcripts was significantly reduced, and in asthma patients. These findings indicate that FoxO transcription factors are relevant in the pathogenesis of chronic inflammatory lung diseases.

2 RESULTS

2.1 Hypoxia induces a dfoxo-dependent immune response in the *Drosophila*

Airways

Hypoxia is a vital airborne stressor that can induce a variety of responses. We have used the fruit fly *Drosophila* as a model to study these responses. To investigate which organs respond most strongly to a hypoxic episode, we used the *LDH-Gal4/UAS-nGFP* reporter strain, which induces nuclear expression of GFP under transcriptional control of the murine LDH-A enhancer and effectively monitors reactions to hypoxia in *Drosophila* (Lavista-Llanos *et al*, 2002). Hypoxia elicited a highly reproducible response in the airway epithelium but not in other organs (**Figure 1A**; background staining of the intestine represents autofluorescence caused by ingested food). This result suggests that the airway epithelium is the primary organ affected by hypoxia. In preliminary experiments, we observed that the epithelial immune system is susceptible to environmental cues. Therefore, we performed a transcript analysis of isolated tracheae, focusing on selected antimicrobial peptides (AMPs). We exposed *Drosophila* larvae to mild hypoxia (5 % O_2 for 2 h) and found that some of the genes studied, namely *cecropin*, *attacin*, and *drosomycin*, were consistently and significantly up-regulated ($p<0.05$; **Figure. 1B**).

We then used the *drosomycin-GFP* (*Drs-GFP*) reporter line to elucidate the spatial dimension of this response. Here, the expression was strongly increased by hypoxic episodes (**Figure 1C**), and the expression appeared first in the most distal part of the airway system, where it was observed in most treated animals (yellow arrows; **Figure 1C**, D). Main branches showed little or no response under these conditions, whereas secondary and terminal branches showed strong fluorescence (red arrows; **Figure. 1C**, D). After prolonged hypoxia, all major branches (except the dorsal trunks) showed robust expression of this antimicrobial (antifungal) peptide gene. In contrast, other organ systems showed no induction of GFP expression in response to hypoxia (**Figure. 1C**, D). When adult animals were exposed to hypoxia, strong GFP expression was observed throughout

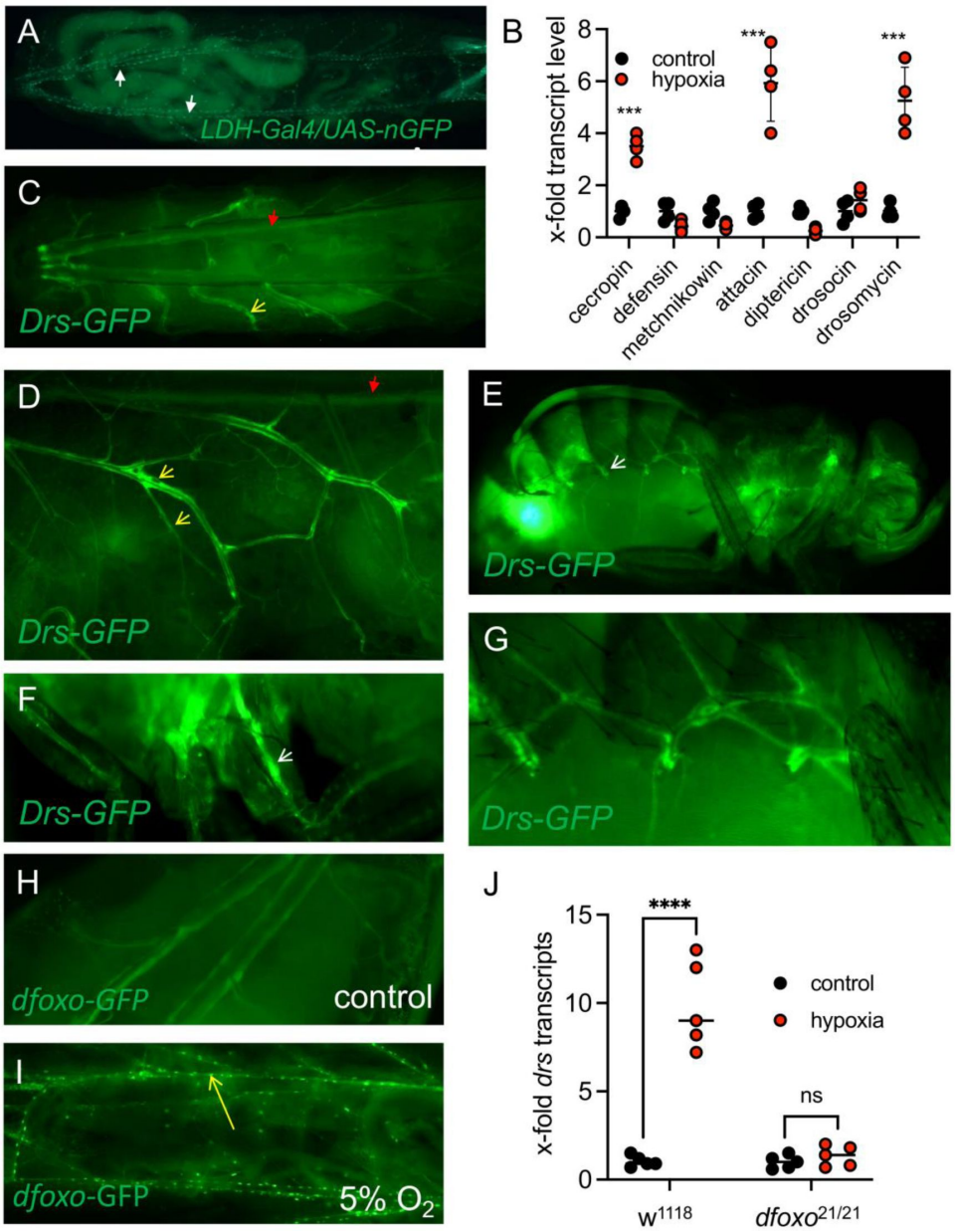

Figure 1

Hypoxia induces a dfoxo-dependent activation of the immune response in *Drosophila* airway epithelia.

A) Early third-instar larvae of the LDH-Gal4/UAS-nGFP genotype were subjected to a brief period of mild hypoxia (2 h, 5% oxygen). Nuclear expression of GFP was observed only in the airway epithelium (arrows). Background staining of the intestine is due to the autofluorescence of ingested material. B) qRT-PCR analysis of the transcript levels of the major antimicrobial peptide (AMP) gene levels in the trachea of larval *Drosophila* in response to a hypoxic episode. C, D) Hypoxia treatment of a *drosomycin* reporter strain (*Drs-GFP*) induced *GFP* expression only in the airway epithelium. C) In the overview, expression is restricted to the primary and secondary branches (yellow arrows), whereas the main trunks show no or little expression (red arrows). D) At higher magnification, expression in the primary and secondary branches is more evident. E-G) Using the same line, hypoxia-induced expression of the *drosomycin* reporter was also observed in the adult tracheal system. E) At low magnification, the peripheral tracheal system is visible through the cuticle (yellow arrows). F) At higher magnification, tracheal structures directly adjacent to the spiracular opening and G) within the legs are visible. *dfoxo-gfp* targeting the tracheal system under H) control and I) hypoxic conditions. J) Hypoxia-induced transcript levels of *drosomycin* in larval trachea of control (left) and *dfoxo*-deficient animals. Mean values ± S.D. ≥ 3, ** p<0.01.

the tracheal system (white arrows, **Figure 1E**). Detailed analysis revealed strong expression in tracheae close to the surface of the animal, namely those in the legs (**Figure. 1F**) and those regions immediately adjacent to the tracheal opening (**Figure 1G**).

To identify the underlying mechanism of this antimicrobial response flies expressing XFP-tagged transcription factors relevant to antimicrobial responses [UAS-*relish-yfp*, UAS-*dorsal-gfp*, UAS-*dif-cfp* (Bettencourt *et al*, 2004), and UAS-*dfoxo-gfp*] were targeted to the airways by crossing with ppk4-Gal4, a tracheal epithelium-specific driver line (Wagner *et al*, 2008). These animals were exposed to hypoxia. Neither Relish nor Dif or Dorsal showed any nuclear translocation following hypoxia. Under normoxic conditions, dfoxo-GFP localizes to the cytoplasm of airway epithelial cells (**Figure 1H**). In response to hypoxia, an almost complete translocation of dFoxo into the nucleus was observed (**Figure 1I**). Even after cessation of hypoxia, dFoxo-GFP remained in the nucleus for some time (usually more than 1h) before re-entering the cytoplasm.

Based on the above results, we tested whether dfoxo is necessary for hypoxia-induced expression of *drosomycin* in the trachea. In *dFoxo*-deficient animals ($dfoxo^{21/21}$), hypoxia-induced *drosomycin* expression was absent, demonstrating that dfoxo is essential for this immune response (**Figure 1J**).

2.2 dfoxo translocation in response to different types of stimuli

Having shown that hypoxia is a major stressor capable of inducing dfoxo translation in the trachea, we investigated to what extent other stressors also induce this response. In parallel, we tested whether the other immune-related transcription factors, Dorsal and Relish, respond with nuclear translation. Ectopically expressed transcription factors labeled with GFP or YFP were introduced into the respiratory tract of *Drosophila* larvae using the Gal4/UAS system (Bettencourt *et al*., 2004). In the untreated control group, Dorsal, Relish, and dfoxo were localized in the cytoplasm (**Figure 2A**, A', A''). Starvation, oxidative stress, UV irradiation, cold stress, and heat stress resulted in a rapid translocation of dfoxo to the nucleus (**Figure 2B-F**). However, nuclear translocation of Relish (**Figure 2B**'-F') and Dorsal (**Figure 2B**''-F'') was not observed, except for a partial nuclear translocation of Relish during starvation stress (**Figure 2B**').

To analyze the underlying activation mechanisms, we also performed these studies when the JNK pathway was knocked down (induced mechanism with Bsk^{DN} by co-expression of the dominant negative JNK allele (Bsk^{DN}), **Fig. 2G-K**'). Under these conditions, the cold-(**Fig. 2H**, H') and heat-induced (**Fig. 2I**, I') translocation of dfoxo was not dependent on the JNK pathway, whereas hypoxia-induced dfoxo translocation was drastically reduced (**Fig. 2K**, K').

2.3 Transcriptomic analyses of *Drosophila* airways upon dfoxo deregulation

We performed transcriptome analyses of isolated *Drosophila* larval airways to learn more about the processes involved in deregulated dfoxo expression. To this end, we analyzed the effects of dfoxo deficiency in $dfoxo^{21/21}$ animals (**Fig. 3A, B**) and those of specific airway-targeted overexpression (**Fig. 3C, D**). *dfoxo* deficiency resulted in the upregulation of 1044 genes and the downregulation of 2133 genes (>1.5-fold, $p<0.05$). In contrast, *dfoxo* overexpression, specifically in the trachea ($dfoxo^{oe}$), resulted in the upregulation of 552 genes and the downregulation of 774 genes. In both experiments, control, and $dfoxo^{21/21}$or $dfoxo^{oe}$ tracheae were significantly separated in the corresponding PCA plots (**Figure 3B**, D). Overlaps between the different groups in the Venn diagram were particularly relevant (**Figure 3E**). We identified 265 genes that were downregulated in *dFoxo*-deficient tracheae and upregulated in $dfoxo^{oe}$ tracheae. Enriched GO terms for biological processes in this cohort were mainly associated with cuticle development and muscle function and development. In addition, 81 genes were downregulated in $dfoxo^{oe}$ flies and upregulated in $dfoxo^{21/21}$ flies (**Fig. 3E**). We also found regulation in the same direction, including those that were downregulated (201 genes) or upregulated (62 genes) in both $dfoxo^{oe}$and

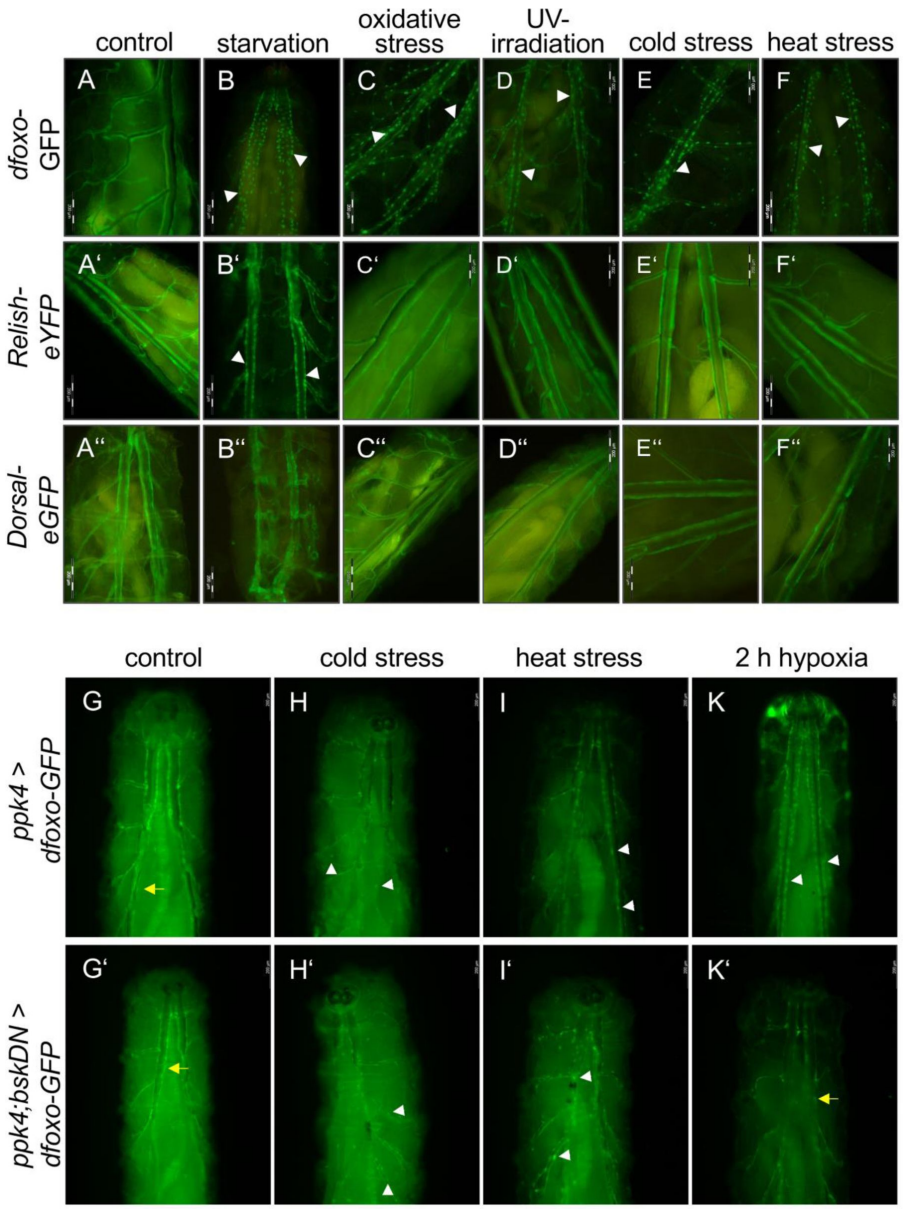

Figure 2

Cellular localizations of the transcription factors dfoxo, relish, and dorsal in the airways of *Drosophila* larvae exposed to different stressors.

A-F) *dfoxo-GFP* was expressed in the airways by crossing the driver line *btl*-Gal4 with the corresponding responder line UAS-*dfoxo-gfp*. A) Under non-stressed conditions, fluorescence was restricted to the cytoplasm. B-F) Starvation (B, 12 h of nutritional deprivation), oxidative stress (C, exposure to 100 mM paraquat for 2 h), UV irradiation (D, 254 nm UVC rays), cold stress (E, 2 h at 4°C), and heat stress (F, 30 min at 41°C) induced nuclear translocation of dFoxo-GFP in epithelial cells of the tracheae. A'-F'') Relish-eYFP and Dorsal-eGFP were expressed by crossing the driver line *btl*-Gal4 with the corresponding responder lines UAS-*relish-eyfp* and UAS-*dorsal-egfp*, respectively. A', A'') Under non-stressed conditions, the fluorescence of Relish-eYFP and Dorsal-eGFP was restricted to the cytoplasm. B', B'') Except for starvation (B'), the various stressors including C') oxidative stress, D') UV irradiation, E') cold stress, F') and heat stress did not induce nuclear translocation of Relish-eYFP. B''-F'') None of the applied stressors induced nuclear translocation of Dorsal-eGFP. G-K) *ppk4-Gal4XUASdfoxo-gfp* animals remained unstressed (G, control), H) experienced cold, I) heat, and K) hypoxia. G'-K') Animals of the genotype *ppk4-Gal4; UAS-bsk^{DN}XUAS-dfoxo-gfp* were kept under control conditions. White arrowheads indicate nuclear translocation. Yellow arrows indicate no nuclear translocation. All images were acquired 2 h, or 12 h in the case of starvation after stress stimulation was initiated. Scale bars are 200 µm.

dfoxo$^{21/21}$ flies. All these overlaps were statistically significant (Fisher's exact test, p<0.05; **Fig. 3E**). Genes downregulated in *dfoxo*$^{21/21}$ flies were particularly interesting. The following KEGG pathways were enriched: various drug-related metabolic detoxification processes, glutathione metabolism, and amino acid metabolism (**Figure 3F**). Transcripts that were upregulated in *dfoxo*$^{21/21}$airways were associated with the following KEGG pathways: DNA replication, Drug metabolism, and associated terms (**Figure 3G**). **Figure 3H** shows those transcripts related to the glutathione metabolism regulated in *dfoxo*$^{21/21}$ airways. In addition, those heat shock proteins (Hsp) that show differential expression (compared to control airways) in *dfoxo*$^{21/21}$ airways are also listed (**Figure 3I**).

2.4 Stress sensitivity of *dfoxo* deficient *Drosophila*

Having shown that various stressors activate dFoxo, we sought to test the hypothesis that dFoxo is essential for an adequate response to stressors. We compared *dfoxo*$^{21/21}$ flies and their control counterparts (yw) after exposure to different stressors (**Figure 4**). It is known that daily exposure to cigarette smoke significantly shortens the lifespan of flies (Prange *et al*, 2018). Our experiment shows that this shortening was greater in *dfoxo*$^{21/21}$ flies compared to control flies exposed to the same protocol (9 days for *dfoxo*$^{21/21}$ flies versus 18 days median lifespan for control flies: **Figure 4A**). Drought stress also reduced the mean lifespan of adult flies. This lifespan-shortening effect was enhanced in *dfoxo*$^{21/21}$ flies (32 h for *dfoxo*$^{21/21}$ flies versus 46 h for control flies: **Figure 4B**). Finally, we examined the effects of hypoxia using a hypoxia-induced larval escape assay (**Figure 4C**) and the hypoxia recovery assay with adult flies (**Figure 4D**). In the former assay, *dfoxo*$^{21/21}$ larvae were more sensitive to hypoxia than control larvae because they left the medium earlier (**Figure 4C**). In the latter assay, the *dFoxo*$^{21/21}$ flies recovered much more slowly than the control flies (**Figure 4D**). Taken together, these experiments demonstrate the increased stress sensitivity of *dfoxo*-deficient animals.

2.5 Effects of hypoxia on hFOXO in human airway epithelial cells

We wondered whether the response to hypoxia observed in *Drosophila* would be similar in the human system. To analyze this, we first investigated whether the different hFOXO factors are expressed in the human lung. We took advantage of the human lung atlas (Vieira Braga *et al*, 2019) and evaluated the expression of the four hFOXO members (**Figure 5A**). hFOXO3 and, to a lesser extent, hFOXO1 and 4 showed broad expression in almost all cell types of the epithelium (**Figure 5A**). In contrast, hFOXO6 is expressed at much lower levels (**Figure 5A**). We used the lung epithelial cell line A549, exposed it to hypoxic conditions, and analyzed the translocation properties of the three major hFOXO factors, hFOXO1, 3, and 4, by immunohistochemistry (**Figure 5B**). hFOXO1 showed a robust nuclear translocation in response to hypoxia (**Figure 5C**), whereas the hypoxia-induced nuclear localization of hFOXO3 and hFOXO4 was less pronounced (**Figure 5C**). We then investigated the extent to which cytokines are also induced by hypoxia in A549 cells. We focused on two cytokines that were shown to react to stressors in airway epithelial cells reproducibly, CCL20 (**Figure 5E**) and CXCL8 (**Figure 5F**)(Guesdon *et al*, 2015; Ovrevik *et al*, 2009; Wolf & Moser, 2012). Both cytokines were significantly upregulated in our experiments in response to hypoxia.

2.5 Stress-induced nuclear translocation of hFOXO factors in human airway epithelial cells

Next, we used human cell lines representing different airway epithelial cell types to evaluate if the stressors identified in the *Drosophila* system also trigger the translocation of hFOXO factors (**Figure 6**). From many different cell lines of the human airway epithelium, we have selected two lines that differ as much as possible in their properties and origins and thus represent a large part of the diversity of these cell lines. One is the A549 line, a cancer cell line representing type 2

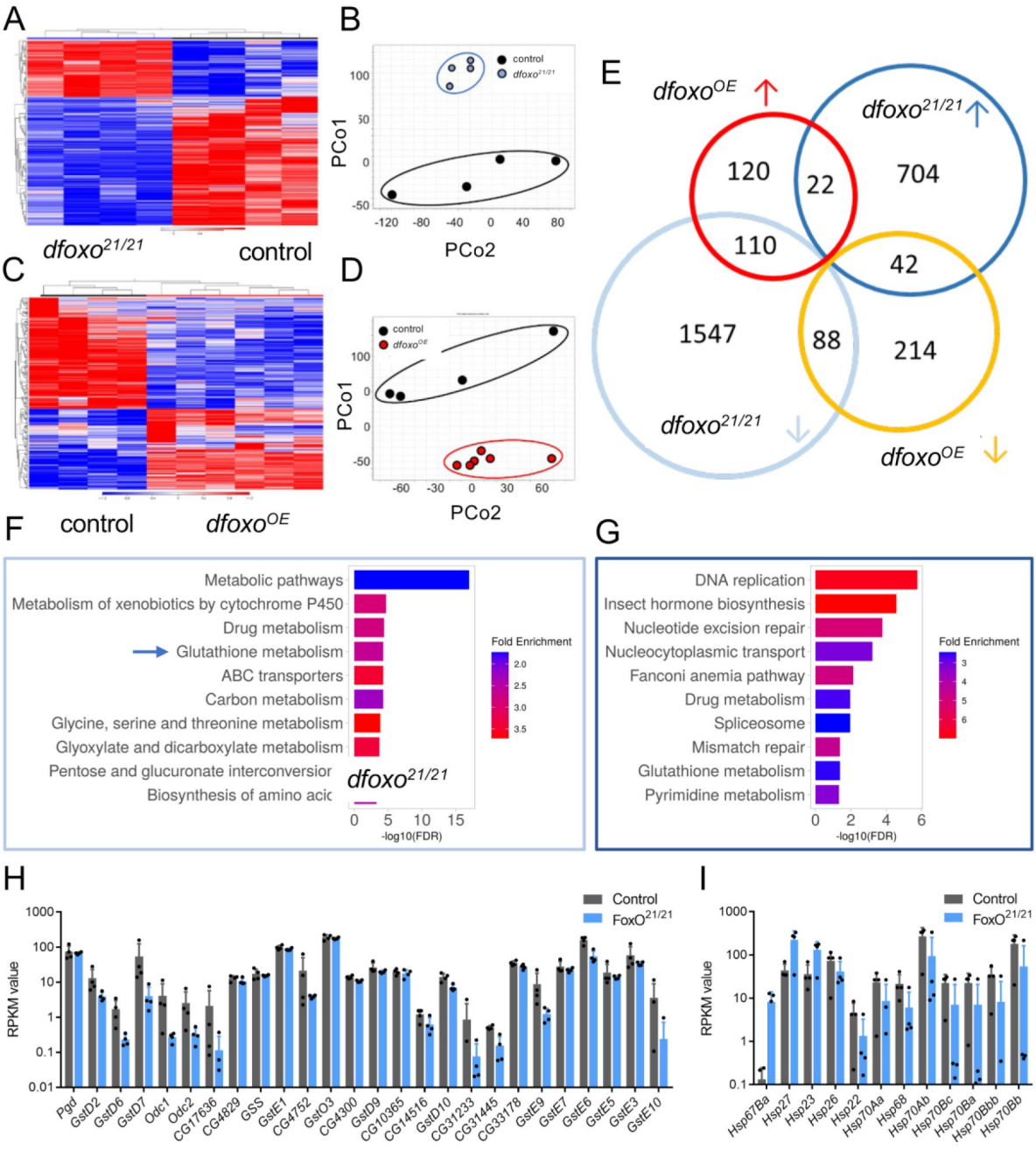

Figure 3

RNAseq analyses of *dfoxo* deficiency and *dfoxo* overexpression in the airways of *Drosophila* larvae.

Heatmap of differentially expressed genes >1.5 fold, ($p<0.05$) of airway samples taken from control and *dfoxo*$^{21/21}$ animals. B) Red indicates upregulation, blue indicates downregulation. PCoA analysis of the individual biological replicates is shown on the right. C) Heatmap of differentially expressed genes between control airways and those experiencing *dfoxo* overexpression (*dfoxo*^{oe}) in the airways. D) PCoA analysis is shown on the right. E) Venn diagram analysis of significantly up- and downregulated genes of comparisons between controls and *dfoxo*$^{21/21}$ as well as controls and *dfoxo* overexpression (oe). The significance of overlapping cohorts of genes was calculated with Fisher's exact test (***$p<0.001$, *$p<0.05$). F) Significantly regulated KEGG pathways in the cohort of genes that are downregulated in *dfoxo*$^{21/21}$. G) Significantly regulated KEGG pathways in the cohort of genes that are upregulated in *dfoxo*$^{21/21}$ trachea. H) Genes that are associated with glutathione metabolism are downregulated in *dfoxo*$^{21/21}$ airways (mean values ± S.D. are shown). I) Heat-shock protein genes whose transcription is differentially regulated in *dfoxo*$^{21/21}$ airways.

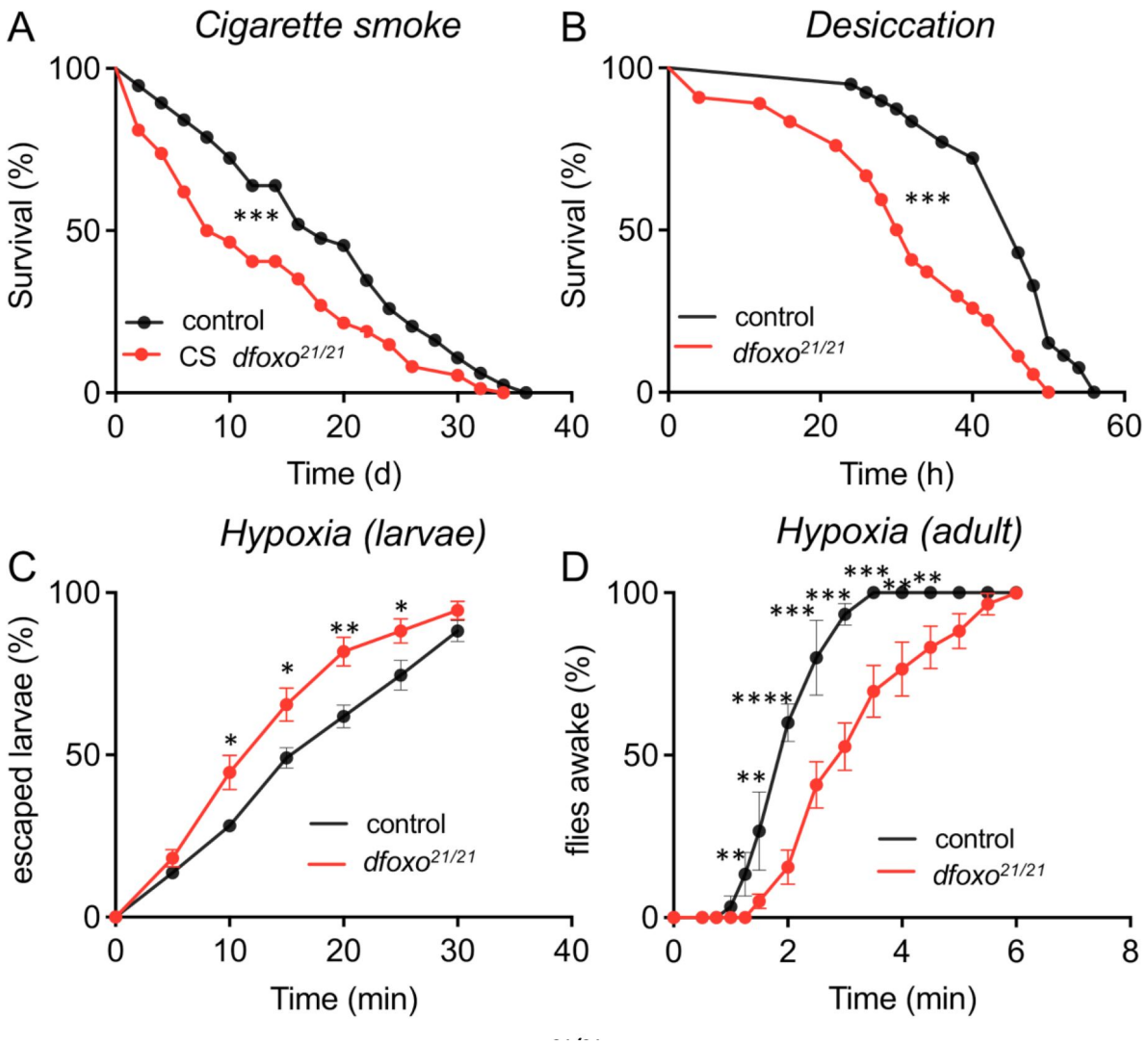

Figure 4

Reduced stress resistance of *dfoxo*$^{21/21}$ flies.

A) Survival of *dfoxo*-deficient (*dfoxo*$^{21/21}$) and control adult flies exposed to cigarette smoke daily (n>10). B) Survival of *dfoxo*-deficient and control flies exposed to desiccation. C) Lawn-leaving assays of *Drosophila* 3rd instar larvae exposed to hypoxia. D) *dfoxo*-deficient and control flies were briefly anesthetized with N_2 and the time taken to full recovery was measured. For A, B, N≥100, for C, D, N=30-110. Shown are mean values ± SEM. * means p<0.05, ** means p<0.01, *** means p<0.005.

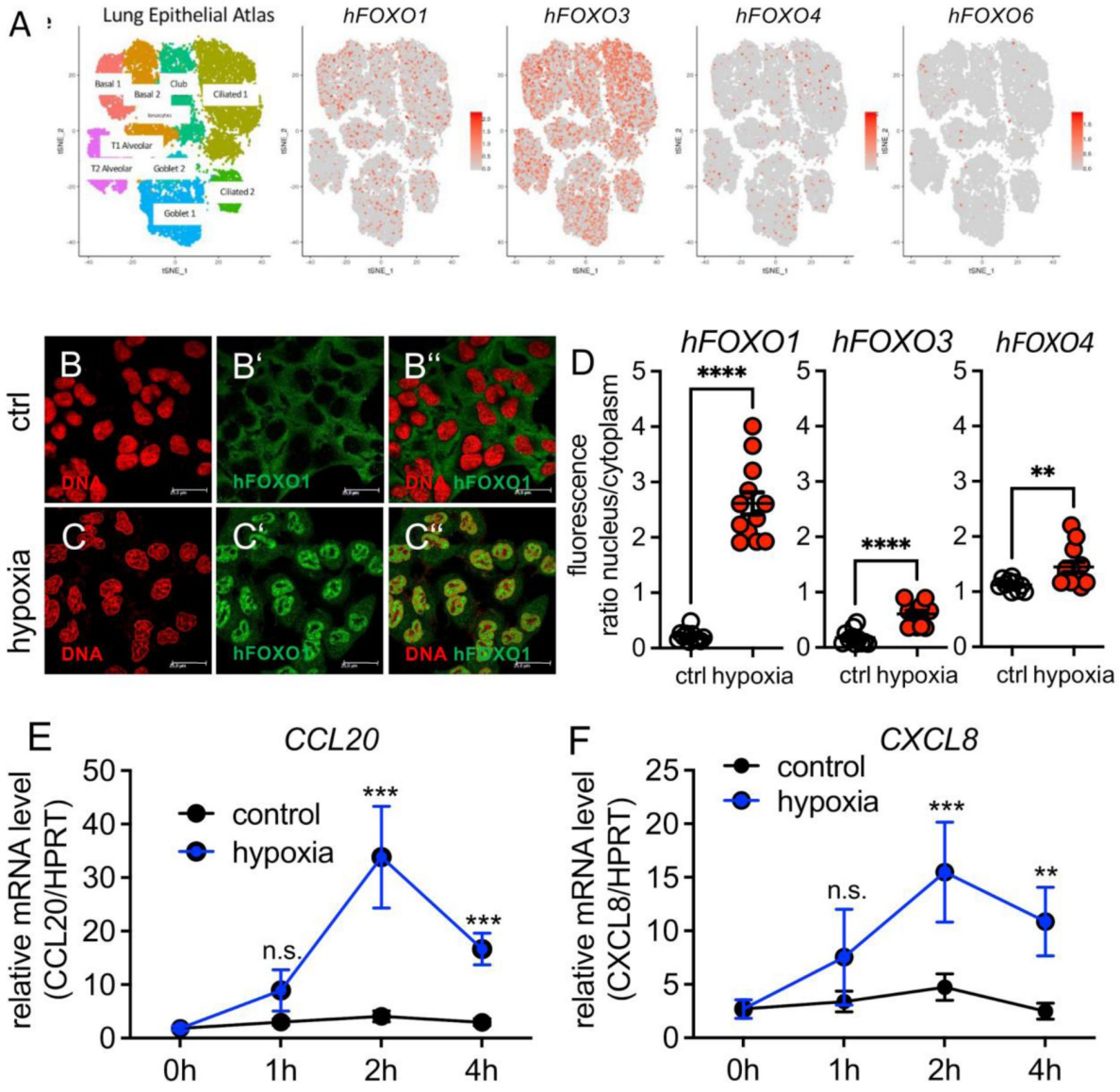

Figure 5

Expression of *hFOXO1*, *hFOXO3A*, *hFOXO4*, and *hFOXO6* in lung tissues of humans and response to hypoxia.

A) Using the human lung atlas (Vieira Braga *et al*., 2019), expression plots of the four different hFOXO genes were generated. Red dots represent positive cells. The assignment to specific epithelial cell types is shown on the left. B-D) Response to hypoxic treatment (5% O_2) in human A549 cells. B) Control cells show a cytosolic localization of hFOXO1, whereas hypoxia induced an almost complete nuclear translocation (C). (D) Quantitative evaluation of hFOXO translocation in response to hypoxia as indicated by hFOXO1, hFOXO3A, and hFOXO4. E, F) Hypoxia-induced expression of cytokines with antimicrobial activities in a time-dependent manner. E) Expression levels of CCL20 in A549 cells treated for different times with hypoxia (5 % O_2). F) The same type of analysis as in D was performed with CXCL8. Data are presented as the mean and SD (n≥7 per group). Statistical analyses were performed using a one-way ANOVA. *p<0.05, **p<0.01, ***p<0.001.

alveolar cells(Foster *et al*, 1998); the other is the BEAS-2B cell line, an immortalized airway epithelial cell line(Fujisawa *et al*, 2000). Under unstressed conditions, all hFOXO proteins (hFOXO1, hFOXO3, hFOXO4, and hFOXO6) were mostly restricted to the cytoplasm in both cell lines (**Figure 6A**, G, H). All tested stressors induced nuclear translocation of hFOXO1 in A549 cells (**Figure 6A-F**). Quantitative analysis of nuclear translocation of hFOXO proteins revealed striking differences between the two cell lines (**Figure 6G**, H). hFOXO1 underwent nuclear translocation in response to all stressors in A549 cells (**Figure 6G**), but only in response to oxidative stress in BEAS-2B cells (**Figure 6H**). hFOXO3A underwent nuclear translocation in response to oxidative stress, starvation, and UV irradiation in A549 cells (**Figure 6G**) but not in response to any stressor in BEAS-2B cells (**Figure 6H**). hFOXO4 (**Figure 6G**, H) and, to a lesser extent, hFOXO6 (**Figure 6G**, H) exhibited highly dynamic nuclear translocation following stress application in both cell lines, suggesting that cells in the upper (BEAS-2B) and lower (A549) airways respond differently to different stressors in terms of nuclear translocation of hFOXO proteins.

To validate this stress-induced nuclear translocation in an *in vivo* organ context, we challenged intact mouse tracheae with paraquat to induce oxidative stress. We focused on mFoxO1, which has shown a robust response to various stressors in cell culture experiments. Most of the mFoxO1 in untreated tracheae was in the cytoplasm and showed nuclear translocation in 52±4 % of the cells (**Fig. 6I-K**). After exposure to paraquat, a massive nuclear translocation of mFoxO1 (**Fig. 6I-K**) was observed in almost all cells (96±0.5 %; p<0.0001).

2.6 FoxO factors are reduced in airway epithelia of murine asthma models and in the sputum of asthmatic patients

So far we have shown that different stressors effectively activate FoxO signaling in the airway epithelia of flies, humans, and mice. Consequently, we asked whether the lung disease that is characterized by intense stress sensitivity, namely asthma, shows altered FoxO signaling in the airways. We analyzed different mouse models of asthma (acute and chronic, **Figure 7**). In the acute asthma model, mice were sensitized to ovalbumin (OVA) (**Figure 7A–D**). We considered the distal and proximal airways separately by physically separating and isolating these airways from each other. There were significant differences between the proximal and distal airways. A distal airway was defined as the segment of a terminal bronchiolus that, starting at the bronchioalveolar duct transition, extended up to five alveoli along the proximal direction (Wegmann *et al*, 2005). In untreated control mice, the relative expression of *mFoxo* factors was higher in proximal airways than in distal airways, and these differences were statistically significant for *mFoxo4* and *mFoxo6* (**Figure 7C**, D). The transcript levels of *mFoxo* genes were always reduced in OVA-treated mice. The transcript levels of *mFoxo1* (**Fig. 7A**) in the proximal airways, *mFoxo4* (**Figure 7C**), and *mFoxo6* (**Figure 7D**) in the proximal and distal airways were significantly lower in OVA-treated mice than in control mice. Finally, we compared whole lung transcript levels between control mice and acute and chronic asthma models (**Figures 7E-H**). *mFoxo* expression was strongly reduced in entire lung tissue from mice with chronic experimental asthma (**Figures 7E-H**). Relative to the transcript levels in lung tissue from healthy mice (set to 100 % in 7E-H), the expression level of *mFoxo1* (**Figure 7E**), *mFoxo3* (**Figure 7F**), *mFoxo4* (**Figure 7G**), and *mFoxo6* (**Figure 7H**) in lungs from mice with chronic experimental asthma went down to 50 %, 47 %, 40 %, and 30 %, respectively, and all these reductions were statistically significant.

Finally, we wanted to see if these findings on the importance of FoxO factors in mouse and *Drosophila* models could also be observed in asthma patients. To this end, we compared the levels of *hFOXO* transcripts in sputum samples from asthmatic patients and healthy individuals. The levels of all four *hFOXO* factors were lower in sputum samples from asthmatic patients than in sputum samples from healthy controls (**Figure 8A**), and these differences were statistically significant for *hFOXO1* and *hFOXO3*. We wanted to know whether *hFOXO* levels are also reduced

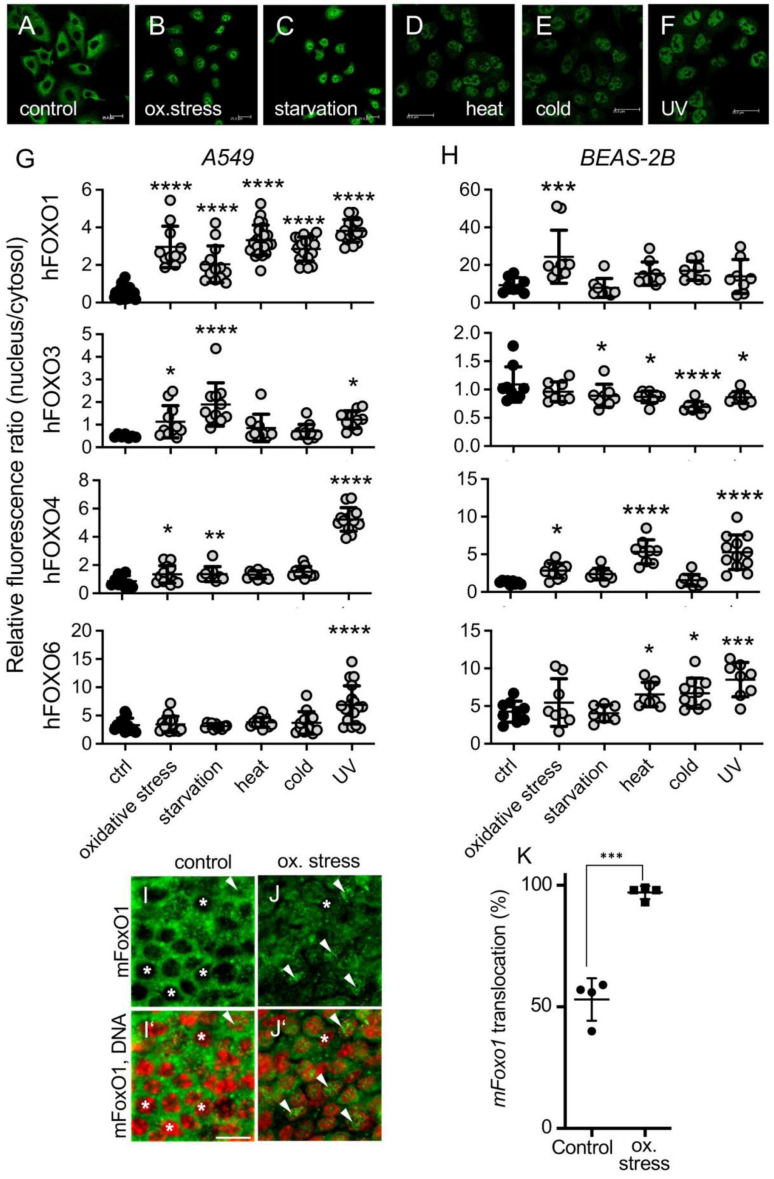

Figure 6

Stress-induced nuclear translocation of FoxO factors in AECs is conserved between species.

A-F) Cultured A549 cells (human alveolar type II cells) were exposed to A) medium only (control) or different stressors, namely, B) oxidative stress induced by paraquat, C) starvation, D) UV light, E) cold stress, and F) heat stress. hFOXO1 was detected immunohistochemically. Scale bars are 25 µm. G-N) Nuclear translocation was quantified by determining the distribution of the antibody-linked fluorescence signal in the nucleus and cytoplasm of G) A549 and H) BEAS-2B cells. Cultured A549 and BEAS-2B (human bronchial epithelial cells) cells were exposed to medium only (ctrl), oxidative stress, starvation, heat stress, cold stress, and UV light. G, H) Immunofluorescence staining revealed the cellular distribution of hFOXO1, hFOXO3A, hFOXO4, and hFOXO6. The relative ratios of fluorescence intensities in the nucleus to those in the cytoplasm are shown. A ratio of 1 indicates that the fluorescence intensities in the cytoplasm and the nucleus are identical. The higher the ratio, the higher the fluorescence intensity in the nucleus. I, J) Semi-intact and freshly explanted mouse tracheae were exposed to medium (control) or paraquat (oxidative stress). Nuclei were stained with Hoechst 33258 (red) and mFoxo1 was immunohistochemically stained (green). I', J') The bottom images are merged. The scale bar is 10 µm. Asterisks indicate nuclear regions without mFoxo1 signal. Arrowheads indicate nuclear regions containing mFoxo1 signals. K) Quantification of nuclear translocation of mFoxo1 in murine tracheae exposed to paraquat. Points represent values obtained in independent experiments/individual animals (n=4). Statistical analysis was performed using the t-test. Significant differences compared with the control were calculated by a one-way ANOVA. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.

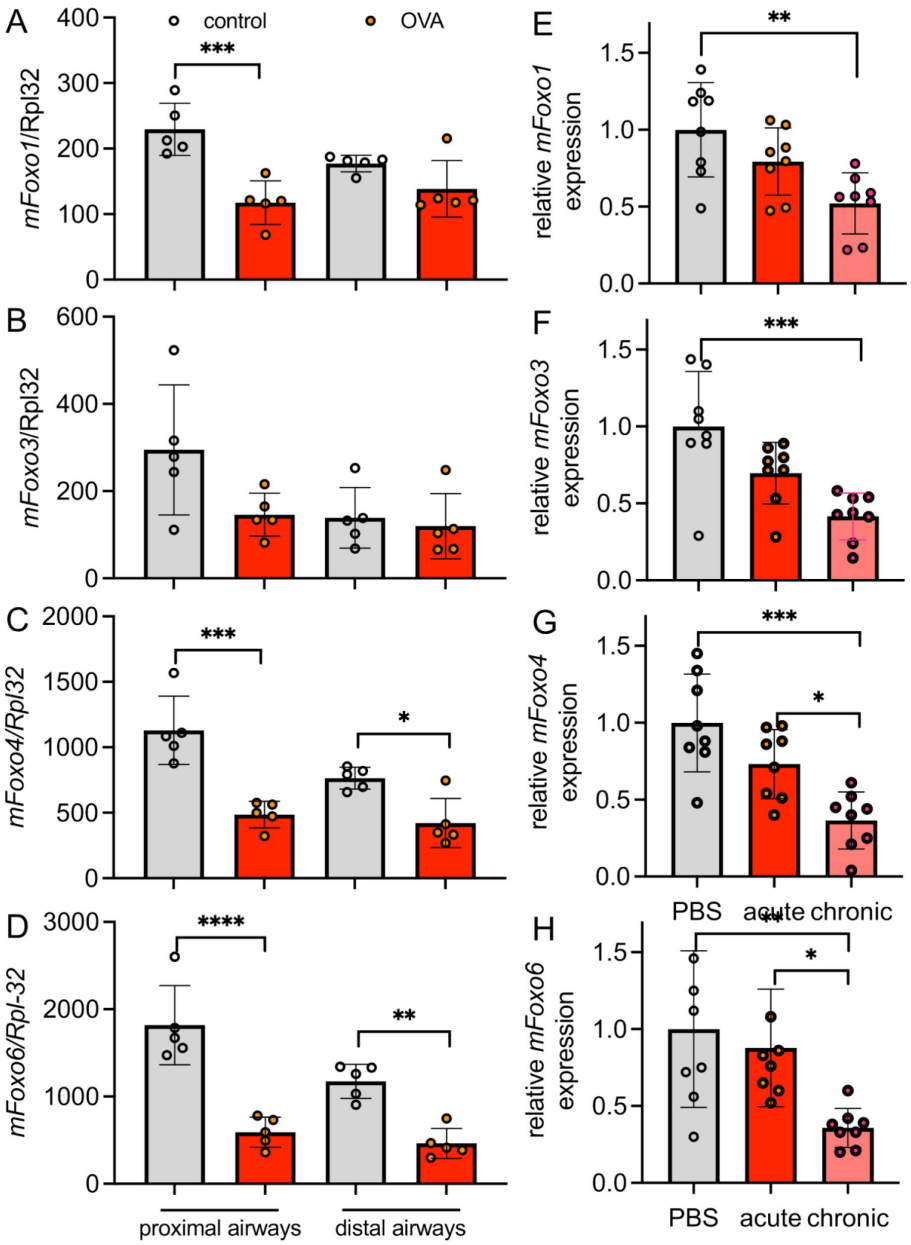

Figure 7

Expression of *mFoxo1*, *mFoxo3A*, *mFoxo4*, and *mFoxo6* in lung tissues of PBS-treated control mice and OVA-treated mice used as models of acute and chronic asthma.

A-D) Micro-dissected airways of PBS-treated control mice and OVA-treated mice (acute asthma model) were separated into distal and proximal parts. A) *mFoxo1*, B) *mFoxo3A*, C) *mFoxo4*, D) and *mFoxo6* were expressed in both lung regions in both types of mice. In PBS-treated mice, expression was higher in proximal airways than in distal airways. Furthermore, expression in both distal and proximal airways was lower in OVA-treated mice than in control mice. However, this reduction was more apparent in proximal airways than in distal airways. In distal airways, expression of A) *mFoxo1,* B) and *mFoxo3* did not significantly differ between control and OVA-treated mice. Data are presented as the mean and SD (n=5). Statistical analysis was performed using a one-way ANOVA. (E-H) Expression of the four *mFoxo* genes was monitored in lung tissues of PBS-treated control mice and OVA-treated mice used as models of acute and chronic asthma. Expression of E) *mFoxo1*, F) *mFoxo3A*, G) *mFoxo4*, H) and *mFoxo6* was reduced in lung tissue of the acute asthma mouse model and was decreased to an even greater extent in lung tissue of the chronic asthma mouse model. Data are presented as the mean and SD (n=4 mice per group). Statistical analyses were performed using a one-way ANOVA. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.

in a matching well-defined experimental model of asthma. For this purpose, we used primary human epithelial cells from healthy donors and quantified the levels of *hFOXO* transcripts under control conditions and following Th2 priming by IL-4 treatment (**Figure 8B**). The transcript levels of all four *hFOXO* factors were strongly reduced in primary NHBEs stimulated with IL-4, and these differences were statistically significant for *hFOXO1*, *hFOXO3*, and *hFOXO6*, which showed reductions of 50 % or more (**Figure 8B**).

We propose that an appropriate FoxO signaling system is necessary to respond adequately to a wide range of stressors in the physiologically normal range, thereby maintaining homeostasis (**Figure 8C**). In asthmatics, however, this system is impaired, meaning that they can no longer react adequately to stressful situations. Thus, the transition from a physiological to a pathophysiological response occurs even in milder stress situations (**Figure 8C**).

3 DISCUSSION

The present work identifies a phylogenetically highly conserved mechanism of cellular response to stressors in airway epithelial cells (AEC). These epithelial cells orchestrate the response of the whole organ to various stressors and thus enable the maintenance of the functional integrity of the entire organ. We showed that this critical task of maintaining a homeostatic situation depends on the activity of FoxO factors in these epithelial cells. Consequently, deregulation of the FoxO system in AECs can be associated with pathological conditions. We have demonstrated reduced levels of FoxO factors in asthma patients and experimental murine asthma models. This association is particularly true for FoxO1, which appears to be affected in the inflamed lungs of both species and has been linked to various diseases (Peng *et al*, 2020). In addition, we have shown that loss of FoxO signalling drastically reduces resistance to airborne stressors. To demonstrate the latter, we deliberately used the simple *Drosophila* model, in which only one dfoxo factor is present, which also fulfills crucial functions in the respiratory epithelium (Wagner *et al*., 2021). The reduced hFOXO activity that we have shown to correlate with asthma in various models also characterizes other chronic lung diseases. In pulmonary hypertension, pathology correlates with decreased hFOXO1 expression (Savai *et al*., 2014). It is also worth noting that deregulation of hFOXO1 in cells of the immune system such as macrophages may contribute to the pathogenesis of asthma (Chung *et al*, 2019). Reduced expression and activity of other hFOXO factors are also associated with lung diseases. For example, reduced hFOXO3A levels correlate with the development of COPD (Ganesan *et al*, 2013; Hwang *et al*., 2011; Pace *et al*, 2016) and IPF (Al-Tamari *et al*., 2018). This disease association is supported as hFOXO3 shows nuclear translocation in AECs from patients suffering from these diseases (Seiler *et al*., 2013). It is also worth mentioning that loss of hFOXO1 and hFOXO3A function is associated with several forms of lung cancer (Gao *et al*, 2018; Mikse *et al*., 2010; Wu *et al*, 2022).

Using the *Drosophila* system, we showed that reduced dfoxo levels and disease-related increased sensitivity to various stressors are causally linked. Following this line of thought, the reduced ability to respond appropriately to multiple stressors due to reduced hFOXO activity could lead to disease development in patients. Studies showing that FoxO factors orchestrate epithelial homeostasis, particularly in response to respiratory stressors and infections, are consistent with this interpretation (Gimenes-Junior *et al*., 2019; Pomies *et al*, 2016). Furthermore, skin epithelia defective in *hFOXO1* expression have a reduced repair capacity (Mori *et al*, 2014), a finding that could also be applied to the lung epithelium. Further evidence supports the interpretation that hFOXO factors play a central role in various lung diseases. The main observation is that a reduced ability to repair damaged epithelia can be a decisive driver of a wide range of lung diseases. This interpretation applies in particular to chronic lung diseases such as COPD and IPF (Barnes *et al*, 2021), in which this reduced ability to repair can be triggered by the

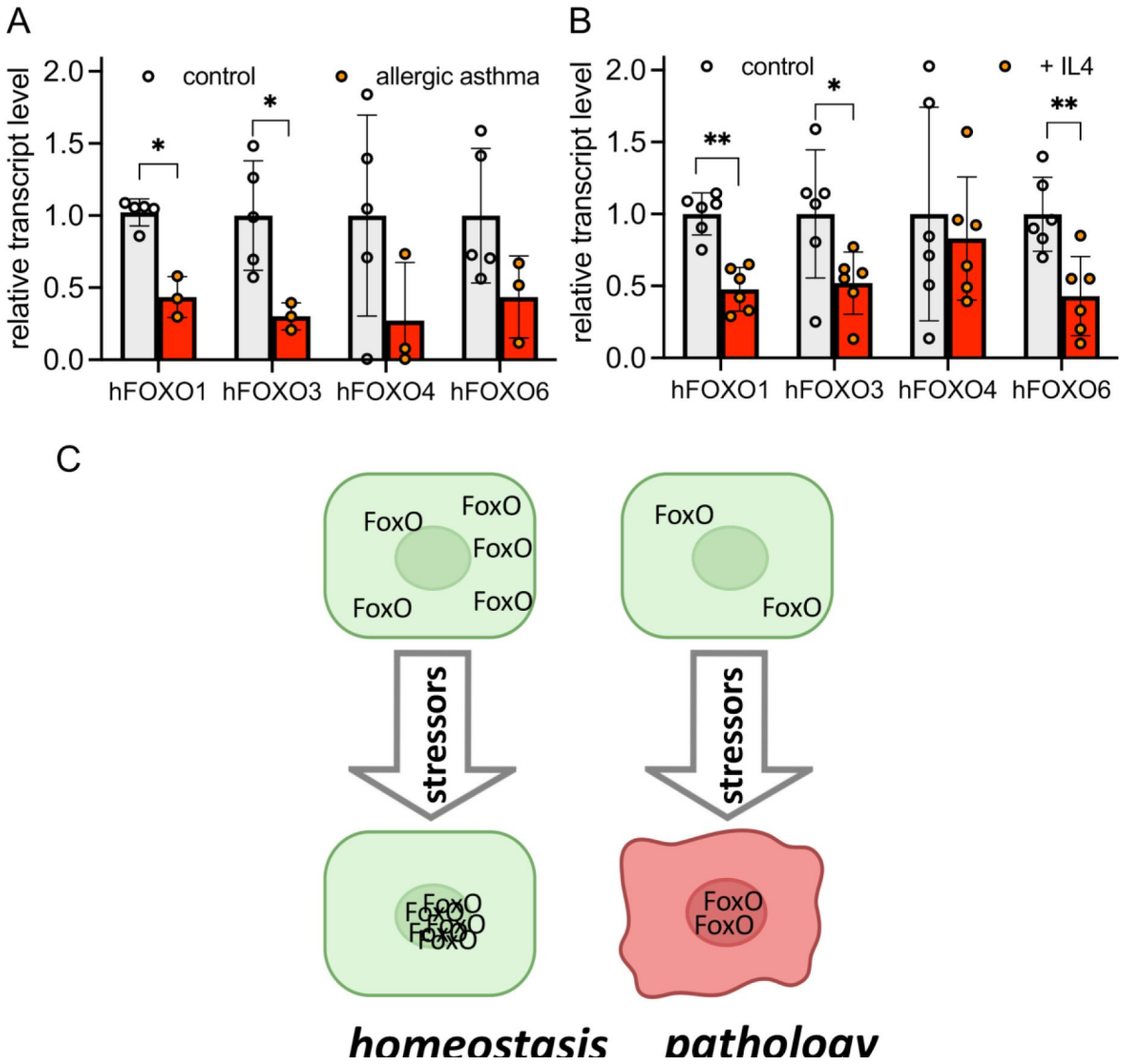

Figure 8

Transcript levels of *hFOXO* factors in sputum samples of asthmatic patients and primary AECs after Th1/Th2 priming.

A) Relative levels of *hFOXO* factors in sputum samples of asthmatic patients and non-asthmatic controls were quantified by mRNA microarray analyses (n≥3). Statistical analysis was performed by the Mann-Whitney U test. B) NHBEs of healthy individuals were cultured under control conditions or treated with IL-4 to induce Th1/Th2 priming. Relative *hFOXO* transcripts were quantified by mRNA microarray analyses (n=6). Statistical analysis was performed by the Mann-Whitney U test. *p<0.05 and **p<0.01. C) Scheme summarizing the function of FoxO factors in a physiological state (left) or in a disease-associated non-physiological state (right).

disease or acquired during the disease. The significance of hFOXO factors for the genesis of these diseases is an exciting topic for future research. Nevertheless, many indications already indicate that hFOXO factors are potential targets for specific interventions.

Our studies on the Th2 polarisation of NHBEs (Zissler *et al*, 2016) indicate a particularly close relationship between asthma and hFOXO factors. The polarization of primary epithelial cells induced by IL-4 treatment reduced the expression of all *hFOXO* factors, like that seen in asthma patients. This result means that the asthma-associated alteration of the airway epithelium, particularly the reduced repair capacity (Ijaz *et al*, 2014), is mediated by the reduced *hFOXO* expression in these cells.

The transcriptome analyses we performed comparing the airways of control animals with those of dfoxo-deficient *Drosophila* revealed a complete network of factors controlled by dfoxo in the airway. Other potential molecular targets associated with a higher prevalence of chronic inflammatory airway diseases are also part of this network. These include heat shock proteins, essential components of the molecular machinery that protects cells from stress (Son *et al*, 2018). The direct link between FoxO activation and the expression of HSP genes thus appears to be a central axis of the cellular stress response. Consequently, disruption of this axis also leads to reduced stress resistance (Donovan & Marr, 2016). In light of this information, it is unsurprising that altered expression of HSPs is associated with asthma (Hou *et al*, 2011). The increased occurrence of relevant HSPs is an excellent indicator of an actual cellular stress situation. On the other hand, reduced HSP activity leads to an inability to respond adequately to this stressful situation, which can then quickly lead to a pathological state. Thus, reduced hFOXO signaling via the impaired hFOXO-HSP axis could increase stress sensitivity (Donovan & Marr, 2016; Kim & Koh, 2017; Tower, 2011). Further studies should address the precise elucidation of this complex, FoxO-dependent network, and its significance for disease development.

The disease implication of deregulated FoxO expression in AECs is a direct consequence of the central physiological role of these transcription factors in adaptation to changing conditions, whether externally or internally induced. The fact that FoxO factors in airway epithelial cells respond to various stressors makes them ideal signaling hubs to adapt the cell response to a stress reaction. The phylogenetic conservation of this stress sensor system makes it possible to analyze its general properties in the simple Drosophila model. Here, we could also determine the causal relationship to reduced stress resistance.

In this study, we have selected a significant number of stressors that, at first glance, do not appear to be equally suitable in all systems but can provide us with information about the general stress response behavior of the AEC. For example, we chose paraquat as an oxidative stress generator because it can be used equally well in all systems (*Drosophila*, mouse, cell culture). Although heat and cold are extremely relevant stressors in *Drosophila*, they are only of limited significance in organisms of the same temperature, such as mice and humans. However, this does not apply to the respiratory air, which can bring such temperature differences to the AEC. In this context, we must realize that not all stressors are mediated via the same signaling pathway to FoxO activation. There is a differential response to the different stressors, particularly with the various hFOXO factors in humans.In contrast to the simple system of a single FoxO factor in *Drosophila*, the human system is complicated because four FoxO factors play this role. This presence of multiple FoxO factors means that the vertebrate system is characterized by high complexity, redundancy, and overlapping functions. In AECs, the four hFOXO factors showed specific response spectra toward different stressors. In particular, hFOXO1 responds very strongly with nuclear translation to various stressors. For the hFOXOs hFOXO3A and hFOXO4, the picture is somewhat more differentiated, as we observed responses to fewer stressors in each case, which also vary strongly between cell types. hFOXO6, on the other hand, generally shows low activation levels but is also expressed at shallow levels. Despite the wealth of information and structural differences leading to different activation modes, our knowledge of the interplay of hFOXO factors still needs to be

more comprehensive (Potente *et al*, 2005; Spreitzer *et al*, 2022). This lack of information concerns the different targets and aspects, such as the complementarity of the action spectra or a functional substitution by compensatory effects. It can be concluded that the human lung is a very complex system in which the different hFOXO factors respond differently to stressors, and this response also differs between the cell types of the epithelium.

Our results show that FoxO factors in the airway epithelium respond to numerous stressors by translocating to the nucleus. In mammals, this response is FoxO factor- and cell type-specific, although there is considerable overlap. This observation suggests the mammalian lung has a highly complex and region-specific stress-sensing system based on FoxO factors. Furthermore, we have shown that a lack of dfoxo expression substantially enhances stress sensitivity, which may underlie the development of several chronic lung diseases. Therefore, FoxO factors and downstream and upstream signaling pathways in AECs are promising therapeutic targets for various chronic lung diseases.

Limitations of the study. We designed this study to identify phylogenetically conserved mechanisms of FoxO activation in airway epithelial cells. For this purpose, we used the experimental systems *Drosophila*, mouse, and patient material to work on the overall project's partial aspects. We used *Drosophila* due to the presence of only one *dfoxo* gene for the general significance of the stress response. The mouse models partially reproduce the response characteristics to analyze acute and chronic asthma models and human material on the one hand for the stress responses of the different hFOXO factors and on the other hand to show the association with asthma using patient material. Although this experimental design provides a comprehensive overall picture, it also means that there are still gaps in the individual experimental systems that need to be closed by follow-up studies. This need for follow-up studies applies in particular to the part of human studies in which we have limited ourselves to available material.

4 MATERIAL AND METHODS

4.1 *Drosophila* stocks, maintenance, and stress assays

Stocks and transgenic crossings were raised on standard cornmeal-agar medium at 21 °C at a relative humidity of at least 60 % in a 12 h:12 h light/dark cycle as described earlier (von Frieling *et al*, 2020; Wagner *et al*., 2021). The following strains used were supplied by the Bloomington and Kyoto/DGGR Stock Center or by scientists of the *Drosophila* research community: *CantonS* (BL1), w^{1118} (BL3605), *Drs-GFP* (BL55707), UAS-bsk^{DN} (BL6409), *btl*-Gal4 (Christian Klämbt, Münster, Germany), *ppk4*-Gal4 (Mike Welsh, Iowa, USA), UAS-*foxo-gfp* (Wagner *et al*, 2009), UAS-*foxo* (BL9575), UAS-*foxo*-TM (Marc Tatar, Brown University, USA), $foxo^{21/21}$ (Ernst Hafen, Zuerich, Switzerland) (Junger *et al*., 2003), UAS-*relish-yfp, LDH-Gal4/UAS-nGFP* (Pablo Wappner, Buenoes Aires, Argentina), and UAS-*dorsal-gfp* (Tony Ip, Massachusetts, USA). To induce expression of either genes of interest or GFP fusion proteins in larval airways we used the binary Gal4/UAS expression system (Brand & Perrimon, 1993). *dFoxo* overexpression experiments were performed using the Target system employing the temperature-sensitive tubP-Gal80ts element (McGuire *et al*, 2004). Therefore, controls were maintained at 18°C, whereas activation was achieved by shifting the temperature to 30°C for 24h.

For all stress tests early 3rd instar larvae were used. Starvation, irradiation, and oxidative stress experiments were done in PBS at 21°C. Starvation experiments proceeded by incubation on PBS-soaked filters for 12 h. For irradiation experiments, larvae were exposed to UV light (254 nm, distance 1 cm) for 15 min with a following incubation in a medium up to 2 h. Oxidative stress was induced using the chemical paraquat (Methyl viologen dichloride hydrate, Sigma-Aldrich, Steinheim, Germany) 100 mM working concentration in a medium enriched solution. Larvae were

incubated in the paraquat solution for 1 h and then transferred to medium for an additional hour. Temperature experiments were performed within the medium. Animals subjected to heat shock were incubated at 37°C for 30 min, translocation experiments were carried out immediately or the larvae remained for 90 min at room temperature. Cold treatment was performed at 4°C for 2 h. Hypoxia (1% O_2) was applied for 4 h under otherwise normal conditions.

Hypoxia induced larval escape assays were performed as shown (Kallsen *et al*, 2015). For the hypoxia recovery assay, animals were briefly (15 s) anesthetized with N_2 and the time to recovery was measured. For the desiccation assay animals were placed into empty vials under constant conditions and survival was monitored. For chronic cigarette smoke exposure, animals were subjected to daily doses of cigarette smoke, and their survival was also monitored daily. For hypoxia experiments, animals were subjected to 5% O_2 for the indicated times. Microscopic analyses regarding *Drosophila* were done *in vivo* with early 3rd instar larvae using a SZX16 stereo microscope and a DP72 camera (Olympus, Hamburg, Germany), and a Zeiss 880 CLSM. Detection of GFP signals was done with an Ex 460-490nm.

4.2 *Drosophila* transcriptomics

Tracheae were manually isolated from 3rd instar larvae and processed as described earlier (Bossen *et al*, 2019; Kallsen *et al*., 2015; Prange *et al*., 2018). The dissected tissue was suspended in RNA Magic (Bio Budget) and homogenized in a bead mill using 1.4 mm zirconia beads (Biolabproducts, Bebensee, Germany). Total RNA input quality was evaluated on a TapeStation 4200 (Agilent, USA), and all samples showed a RIN score > 8. Samples were quantified with a fluorometric dye (Quant-IT, Thermofisher, USA) and between 187 and 500 ng per sample were used as input for the TruSeq stranded mRNA library kit (Illumina, USA) following the manufacturers manual. Resulting libraries showed a fragment size distribution of around 350 bp and were sequenced on a HiSeq 4000 (Illumina, USA) with 50bp single-end reads. Differential expression of sequencing data (GSE176540) was performed with the CLC genomic workbench software (*http://www*. clcbio.com/products/clc-genomics-workbench) (Qiagen, Hilden, Germany), using the *Drosophila melanogaster* reference genome (Release 6) (Hoskins *et al*, 2015). GO analyses were conducted by using the g:Profiler tool (Raudvere *et al*, 2019).

4.3 Cell culture (human-derived cell lines) and stress assays

The alveolar epithelium cell line A549 and the bronchial epithelial cell line BEAS-2B were cultured in RPMI (Biochrom, Berlin, Germany) supplemented with 10 % fetal calf serum, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C with 5 % CO_2. Stress experiments were performed in µ-Slides 8 well ibiTreat (Ibidi, Martinsried, Germany) with $2{,}5\times10^4$ cells per well, cells adhered to the slides overnight. Medium was replaced with either pure RPMI (unsupplemented control), RPMI with paraquat [20 mM] (oxidative stress) or PBS (starvation) and incubated for 1 h at 37°C with 5 % CO_2. Induction of cold and heat stress took place for 1 h each at 41°C and 4°C, respectively. Exposure to UV light (254 nm, distance 1 cm) took place for 15 min with a following incubation up to 1 h. Hypoxic conditions were effectuated in a supplemented medium for 2 h and 5 % O_2, by discharging of N_2 into a gas-tight chamber. Immediately afterward cells were fixed with 3 % paraformaldehyde, permeabilized with 0,25 % Triton-X/PBS, blocked with 10 % BSA/PBS and incubated with primary antibodies: anti-FoxO1A antibody (rabbit polyclonal ChIP Grade), anti-FoxO3A antibody (rabbit polyclonal), anti-FoxO4 antibody (rabbit polyclonal); (abcam, Cambridge, UK) or anti-FoxO3 antibody (rabbit polyclonal); (Thermo Sci. / Pierce Biotech, Rockford, USA), secondary antibody: Alexa Fluor 488 goat anti-mouse IgG (Invitrogen, Karlsruhe, Germany). Nuclei were stained with Bisbenzimide H 33342 (Sigma-Aldrich, Deisenhofen, Germany).

Human cells (A549 and BEAS-2B) were investigated using the TCS Sp5 Inverted Confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany) and a 20x /0.70 HC Plan Apochromat, oil-objective. Antibody and Hoechst-signals were detected with excitation lines of 488 and 405 nm, respectively. All editing procedures were carried out with the Leica LAS AF Lite

program. Quantification of the nuclear translocation in the A549 and BEAS-2B was performed using ImageJ software (Wayne Rasband, NIH). Randomly selected cells were photographed, and all images were taken with constant settings. Considering the area size, the average fluorescence intensity of the nucleus and cytoplasm was determined. The ratio between nucleus and cytoplasm are shown graphically and compared to the ratio of untreated control cells.

Relative quantification of human cytokine transcripts of CCL20 and CXCL8 (IL8) was performed by real-time PCR. Total RNA (A549 cells; standard incubation control and after 1,2,4 h at hypoxic conditions) was extracted using the NucleSpin RNA Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. 500 ng of each RNA sample was transcribed into cDNA, using Oligo dt 12-18 primer mix, RNAse OUT, dNTP mix and SuperScript reverse transcriptase (Invitrogen, Darmstadt, Germany)). The human HPRT gen served as reference für relative expression. Statistical analysis: rato paired t-test, CCL20: n=7, CXCL8 n=6.

4.4 Detection of mFoxO1 nuclear translocation in AECs of the murine trachea

Three-month-old C57BL/6 female mice were used. All experiments were performed in accordance with the government guidelines for animal welfare of the Land Schleswig-Holstein. Mice were killed by an overdose of isoflurane and the trachea was explanted, opened along the tracheal muscle, and cut in half. One half was incubated for 30 min at 37 °C in 100 mM paraquat (Sigma-Aldrich, Taufkirchen, Germany) prepared in HEPES-buffered Ringer solution. The other half was incubated in HEPES-buffered Ringer solution lacking paraquat. Thereafter, the tissue was fixed with PBS containing 4 % paraformaldehyde and processed for immunohistochemistry with an anti-FoxO1 antibody (1:1000 in TBS, Thermo Fisher Scientific, Rockford, USA) at room temperature overnight followed by Alexa Fluor 555-conjugated donkey anti-rabbit IgG (1:500 in TBS, Invitrogen, Karlsruhe, Germany) for 1 h at room temperature and then Hoechst 33258 (1:10,000 in TBS; Sigma-Aldrich GmbH, Taufkirchen, Germany) for 10 min. Three-dimensional confocal image stacks of the epithelial layer were acquired and processed using the ImageJ "Cell Counter" Plugin. AECs were counted in 4 stimulated and 4 control pieces of the trachea. Approximately 200 nuclei were analyzed per piece.

4.5 Quantification of gene expression in mouse lung tissue

Animal experiments were performed using female C57BL/6J (Charles River, Sulzfeld, Germany) mice for expression analysis in proximal and distal airways and for expression analysis in acute and chronic asthma models. All animal studies were approved by the local animal ethics committee (for the acute asthma model: 13-3/20, for the chronic asthma model: 55-6/20; Behörde das Ministerium für Landwirtschaft, ländliche Räume, Europa und Verbraucherschutz Schleswig-Holstein).

Mice were subjected only to the standard mouse protocol for acute experimental allergic asthma (Bulek *et al*, 2009), while the other mice were exposed to the protocols for both acute and chronic experimental allergic asthma (Wegmann *et al*., 2005). In brief, mice were first systemically sensitized against ovalbumin (OVA) by intraperitoneal (i.p.) injection of 10 µg dissolved OVA in 100 µl PBS and 100 µl aluminium hydroxide as adjuvant on day 1, 14 and 21. On days 26-28, animals were exposed to OVA aerosol (1% OVA dissolved in PBS) in an airtight chamber for 20 min. For the chronic asthma model the sensitization was continued twice weekly for 12 weeks on. Control animals received an i.p injection with PBS on the same days and were treated with a PBS aerosol likewise. Lungs were filled with 600 µl RNAlater (Thermo Scientific, Darmstadt, Germany) dissected, and lungs were divided into proximal and distal parts if necessary. The tissue was transferred into RNA lysis buffer, snap-frozen in liquid nitrogen, and homogenized mechanically with the addition of liquid nitrogen. RNA isolation was performed from 30 mg using the RNeasy Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA concentration and purity were determined using a nanophotometer. Reverse transcription of

the isolated RNA into cDNA was performed using the Maxima First Strand cDNA Synthesis Kit (Thermo Scientific, Darmstadt, Germany) according to the instructions of the manufacturer. Gene expression was determined by qRT-PCR on Light Cycler 480II (Roche, Mannheim, Germany) using 2.5 µl cDNA, SYBR Green Mastermix (Roche, Mannheim, Germany), and 10 pmol/µl of each primer. Expression of the gene rpl32 was used as a reference. The sequences of the primers were as follows: rpl32: for 5'-AAAATTAAGCGAAACTGGCG-3', rev 5'-GAAGATGGTGATGGGCTTCC-3'; mfoxO1: for 5'-CTTCAAGGATAAGGGCGACA-3', rev 5'-GACAGATTGTGGCGAATTGA-3'; mfoxO3a: for 5'-GCTAAGCAGGCCTCATCTCA-3', rev 5'-TTGTGTCAGTTTGAGGGTCT-3'; mfoxO4: for 5'-TGTGCTCGCATCTCCTACTG-3', rev 5'-GACTCAGGGATCTGGCTCAAA-3'; mfoxO6: for 5'-CAAGAGACTCACGCTCTCGC −3', rev 5'-GCCGAATGGAGTTCTTCCAGC-3'.

4.6 Human study subjects, lung function testing, sputum sampling, and primary cell culture

Healthy subjects (n=12) and mild, well-controlled, allergic asthma patients along the GINA definition in season (n=9) in good health (FEV1% >70%) with a history of clinically significant hay fever during the grass-pollen season since more than two years were included. For asthmatic subjects, a GINA classification was performed. All subjects completed a Rhinoconjunctivitis Quality of Life Questionnaire (RQLQ), a lung function test, and a hypertonic saline sputum induction, performed in the Allergy Section, Department of Otolaryngology, TUM School of Medicine. Each participant provided written informed consent. This study was approved by the local ethics committee (5534/12). Baseline lung function was evaluated using a calibrated handheld pulmonary function testing device (Jaeger SpiroPro; Würzburg, Germany). The following parameters were recorded: VC, FEV1, FEV1/VC, and maximum expiratory flow 25% (MEF 25%). Bronchodilatator reversibility was tested after 400 µg of salbutamol.

Participants first inhaled salbutamol and consecutively nebulized hypertonic saline at increasing concentrations of 3%, 4%, and 5% NaCl every 7 min according to previous publications. During this procedure, participants cleaned their noses and rinsed their mouth to reduce squamous epithelium cells in the samples. Sputum was processed within one hour of collection. The selected sputum plugs, which contained as little saliva as possible were placed in a weighed Eppendorf tube and processed with 4x weight/volume of sputolysin working solution (Merck, Darmstadt, Germany). Afterwards, 2x weight/volume of phosphate-buffered saline (PBS) was added. Samples were filtered through a 70-µm mesh and centrifuged for 10 min at 790xg without break to remove the cells. Appropriate sputum samples were obtained from 12 healthy controls and 9 asthma patients during the grass-pollen season.

Primary NHBEs (Lonza, Walkersville, MD) of six genetically independent donors were grown as monolayers in 100% humidity and 5% CO2 at 37 1C in serum-free defined growth media (BEGM, Lonza). NHBEs (passage 3) were used at about 80% confluence in 6-well plates. To avoid gene expression changes or influences on the IL-4 signaling induced by growth factors in the BEGM medium, cells were rested in basal medium (BEBM) for 12 h, then stimulated with recombinant human IL-4 at 50 ng/mL (R&D Systems, Minneapolis, MN) in BEBM medium for 6 h or BEBM medium as control condition. For RNA analysis, harvested cells were lysed in RLT buffer (Qiagen, Hilden, Germany) containing 1% beta-mercaptoethanol (Roth, Karlsruhe, Germany) directly in the cell culture well.

Total RNA was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany) with on-column DNase digestion (Qiagen) for avoiding DNA contaminations. RNA quantification was performed by ultraviolet–visible spectrophotometry (Nanodrop Technologies, Wilmington, DE), for assessment of the RNA integrity by the RNA 6000 Nano Chip Kit with the Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany), and samples with RIN number above 7.5 were used for micro-array analysis. Total RNA was amplified and Cy3-labeled by using the one-color Low Input Quick Amp Labeling Kit (Agilent Technologies) according to the manufacturer's protocol.

Hybridization to SurePrint G3 Human Gene Expression 8×60K Microarrays (Agilent Technologies) was performed with the Gene Expression Hybridization Kit (Agilent Technologies) as described before.

Data import using a standard baseline transformation to the median of all values was performed, including log transformation and computation of fold changes. Subsequently, a principle component analysis (PCA) was conducted and revealed a homogenous component distribution. Compromised array signals (array spot is non-uniform if pixel noise of feature exceeds threshold or above saturation threshold) were excluded from further analysis. For this analysis, we set the normalized values of each healthy donor in sputum samples to 1 and calculated the difference of *FoxO1*, *FoxO3*, *FoxO4,* and *FoxO6* expression in sputum of the asthma patient. For analysis of *FoxO1*, *FoxO3*, *FoxO4,* and *FoxO6* expression in primary normal human bronchial epithelial cells (NHBEs), we set the normalized values of medium conditions (in NHBEs) to 1 and calculated the difference in abundance of the IL-4 stimulated condition. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO) and are accessible through GEO Series accession number GSE167225.

For RNA quality assessment, RNA integrity numbers (RIN) were measured via Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). After quality assessment, isolated total RNA was subjected to reverse transcription using a high-capacity cDNA kit (Applied Biosystems, USA), following the manufactureŕs instructions. Real-time PCR profiles were visualized using the commercially available FastStart Universal SYBR Green Mastermix (Roche, SUI) and Qiagen human Primer Assays (Qiagen, Hilden, Germany) specific for *FoxO1* and *FoxO3* (the latter only in NHBEs), and quantified by the ViiA 7 Real-Time PCR System (Applied Biosystems, USA). The amount of *FoxO1* and *FoxO3* mRNA expression was normalized with endogenous control *TFRH*, *GAPDH* and *ß-actin* (housekeeping gene index, DCt values) and the relative quantification and calculation of range of confidence was performed using the comparative threshold cycle (2^{-DDCt}) method (relative gene expression). All amplifications were carried out at least in duplicate.

All experimental procedures and analyses were conducted by blinded research staff. Non-parametric Mann–Whitney U tests were used to determine significant differences between patient groups. All statistical tests were performed two-sided.

Author contributions

T.R., H.H., and P. I. P. conceived the project. T.R., H.H., P.I.P., I.B., K.U., C.S.W., P.K. wrote the manuscript. K.U., J.B., X.N., U.Z. R.P. C.F., M.P. C.V., C.W., S.F., and A.A. performed all experiments, analyzed the data, and made the figures.

Acknowledgements

This work was supported by grants from the Deutsche Forschungsgemeinschaft (DFG) as part of the CRCs TR22 (TPA7) and 1182 (TPC2) and INST 257/591-1 FUGG as well as from the University Kiel in the program of the LeibnizCampus *EvoLung*.

Conflict of interest statement

All authors declare no conflict of interest.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID IDs

Karin Uliczka: 0009-0008-2114-6179

Judith Bossen: 0000-0002-6604-4570

Ulrich Zissler: 0000-0003-4826-3419

Christine Fink: 0000-0003-0083-2168

Xiao Niu: 0000-0001-5246-1376

Mario Pieper: 0000-0003-2589-6782

Ruben D. Prange: 0000-0001-6941-4509

Christina Vock: 0000-0003-3936-3426

Christina Wagner: 0009-0005-8121-0046

Mirjam Knop: 0009-0002-2530-9701

Ahmed Abdelsadik: 0000-0002-4782-6681

Sören Franzenburg: 0000-0001-6374-4910

Iris Bruchhaus: 0000-0002-3363-7409

Michael Wegmann: 0000-0002-1658-1554

Peter König: 0009-0000-7637-7451

Carsten Schmidt-Weber: 0000-0002-3203-8084

Petra Pfefferle: 0000-0002-3102-3737

Holger Heine: 0000-0001-8128-5068

Thomas Roeder: 0000-0002-3489-3834

References

Al-Tamari HM *et al.* (2018) **FoxO3 an important player in fibrogenesis and therapeutic target for idiopathic pulmonary fibrosis** *EMBO Mol Med* **10**:276–293

Barnes PJ, Anderson GP, Fageras M, Belvisi MG (2021) **Chronic lung diseases: prospects for regeneration and repair** *Eur Respir Rev*

Becker T, Loch G, Beyer M, Zinke I, Aschenbrenner AC, Carrera P, Inhester T, Schultze JL, Hoch M (2010) **FOXO-dependent regulation of innate immune homeostasis** *Nature* **463**:369–373

Bettencourt R, Asha H, Dearolf C, Ip YT (2004) **Hemolymph-dependent and-independent responses in Drosophila immune tissue** *J Cell Biochem* **92**:849–863

Bossen J *et al.* (2019) **An EGFR-Induced Drosophila Lung Tumor Model Identifies Alternative Combination Treatments** *Mol Cancer Ther* **18**:1659–1668

Brand AH, Perrimon N (1993) **Targeted gene expression as a means of altering cell fates and generating dominant phenotypes** *Development* **118**:401–415

Bulek K *et al.* (2009) **The essential role of single Ig IL-1 receptor-related molecule/Toll IL-1R8 in regulation of Th2 immune response** *J Immunol* **182**:2601–2609

Burgoyne RA, Fisher AJ, Borthwick LA (2021) **The Role of Epithelial Damage in the Pulmonary Immune Response** *Cells*

Sozener Z Celebi, Cevhertas L, Nadeau K, Akdis M, Akdis CA (2020) **Environmental factors in epithelial barrier dysfunction** *J Allergy Clin Immunol* **145**:1517–1528

Cheng Z (2019) **The FoxO-Autophagy Axis in Health and Disease** *Trends Endocrinol Metab* **30**:658–671

Chung KF, Adcock IM (2008) **Multifaceted mechanisms in COPD: inflammation, immunity, and tissue repair and destruction** *Eur Respir J* **31**:1334–1356

Chung S *et al.* (2019) **FoxO1 is a critical regulator of M2-like macrophage activation in allergic asthma** *Allergy* **74**:535–548

Donlon TA *et al.* (2022) **FOXO3, a Resilience Gene: Impact on Lifespan, Healthspan, and Deathspan** *J Gerontol A Biol Sci Med Sci* **77**:1479–1484

Donovan MR, Marr MT (2016) **dFOXO Activates Large and Small Heat Shock Protein Genes in Response to Oxidative Stress to Maintain Proteostasis in Drosophila** *J Biol Chem* **291**:19042–19050

Fink C, Hoffmann J, Knop M, Li Y, Isermann K, Roeder T (2016) **Intestinal FoxO signaling is required to survive oral infection in Drosophila** *Mucosal Immunol* **9**:927–936

Flachsbart F, Caliebe A, Kleindorp R, Blanche H, von Eller-Eberstein H, Nikolaus S, Schreiber S, Nebel A (2009) **Association of FOXO3A variation with human longevity confirmed in German centenarians** *Proc Natl Acad Sci U S A* **106**:2700–2705

Foster KA, Oster CG, Mayer MM, Avery ML, Audus KL (1998) **Characterization of the A549 cell line as a type II pulmonary epithelial cell model for drug metabolism** *Exp Cell Res* **243**:359–366

Fujisawa T, Kato Y, Atsuta J, Terada A, Iguchi K, Kamiya H, Yamada H, Nakajima T, Miyamasu M, Hirai K (2000) **Chemokine production by the BEAS-2B human bronchial epithelial cells: differential regulation of eotaxin, IL-8, and RANTES by TH2-and TH1-derived cytokines** *J Allergy Clin Immunol* **105**:126–133

Ganesan S, Unger BL, Comstock AT, Angel KA, Mancuso P, Martinez FJ, Sajjan US (2013) **Aberrantly activated EGFR contributes to enhanced IL-8 expression in COPD airways epithelial cells via regulation of nuclear FoxO3A** *Thorax* **68**:131–141

Gao Z, Liu R, Ye N, Liu C, Li X, Guo X, Zhang Z, Li X, Yao Y, Jiang X (2018) **FOXO1 Inhibits Tumor Cell Migration via Regulating Cell Surface Morphology in Non-Small Cell Lung Cancer Cells** *Cell Physiol Biochem* **48**:138–148

Giannakou ME, Goss M, Jacobson J, Vinti G, Leevers SJ, Partridge L (2007) **Dynamics of the action of dFOXO on adult mortality in Drosophila** *Aging Cell* **6**:429–438

Gimenes-Junior J, Owuar N, Vari HR, Li W, Xander N, Kotnala S, Sajjan US (2019) **FOXO3a regulates rhinovirus-induced innate immune responses in airway epithelial cells** *Sci Rep* **9**

Graves DT, Milovanova TN (2019) **Mucosal Immunity and the FOXO1 Transcription Factors** *Front Immunol* **10**

Greer EL, Brunet A (2005) **FOXO transcription factors at the interface between longevity and tumor suppression** *Oncogene* **24**:7410–7425

Guesdon W *et al.* (2015) **CCL20 Displays Antimicrobial Activity Against Cryptosporidium parvum, but Its Expression Is Reduced During Infection in the Intestine of Neonatal Mice** *J Infect Dis* **212**:1332–1340

Gui T, Burgering BMT (2022) **FOXOs: masters of the equilibrium** *FEBS J* **289**:7918–7939

Heijink IH, Kuchibhotla VNS, Roffel MP, Maes T, Knight DA, Sayers I, Nawijn MC (2020) **Epithelial cell dysfunction, a major driver of asthma development** *Allergy* **75**:1902–1917

Hellings PW, Steelant B (2020) **Epithelial barriers in allergy and asthma** *J Allergy Clin Immunol* **145**:1499–1509

Hewitt RJ, Lloyd CM (2021) **Regulation of immune responses by the airway epithelial cell landscape** *Nat Rev Immunol* **21**:347–362

Hornsveld M, Dansen TB, Derksen PW, Burgering BMT (2018) **Re-evaluating the role of FOXOs in cancer** *Semin Cancer Biol* **50**:90–100

Hoskins RA *et al.* (2015) **The Release 6 reference sequence of the Drosophila melanogaster genome** *Genome Res* **25**:445–458

Hou C, Zhao H, Li W, Liang Z, Zhang D, Liu L, Tong W, Cai SX, Zou F (2011) **Increased heat shock protein 70 levels in induced sputum and plasma correlate with severity of asthma patients** *Cell Stress Chaperones* **16**:663–671

Hwang JW, Rajendrasozhan S, Yao H, Chung S, Sundar IK, Huyck HL, Pryhuber GS, Kinnula VL, Rahman I (2011) **FOXO3 deficiency leads to increased susceptibility to cigarette smoke-induced inflammation, airspace enlargement, and chronic obstructive pulmonary disease** *J Immunol* **187**:987–998

Ijaz T, Pazdrak K, Kalita M, Konig R, Choudhary S, Tian B, Boldogh I, Brasier AR (2014) **Systems biology approaches to understanding Epithelial Mesenchymal Transition (EMT) in mucosal remodeling and signaling in asthma** *World Allergy Organ J* **7**

Jiramongkol Y, Lam EW (2020) **FOXO transcription factor family in cancer and metastasis** *Cancer Metastasis Rev* **39**:681–709

Junger MA, Rintelen F, Stocker H, Wasserman JD, Vegh M, Radimerski T, Greenberg ME, Hafen E (2003) **The Drosophila forkhead transcription factor FOXO mediates the reduction in cell number associated with reduced insulin signaling** *J Biol* **2**

Kallsen K, Zehethofer N, Abdelsadik A, Lindner B, Kabesch M, Heine H, Roeder T (2015) **ORMDL deregulation increases stress responses and modulates repair pathways in Drosophila airways** *J Allergy Clin Immunol* **136**:1105–1108

Kim S, Koh H (2017) **Role of FOXO transcription factors in crosstalk between mitochondria and the nucleus** *J Bioenerg Biomembr* **49**:335–341

Lambrecht BN, Hammad H (2012) **The airway epithelium in asthma** *Nat Med* **18**:684–692

Lavista-Llanos S, Centanin L, Irisarri M, Russo DM, Gleadle JM, Bocca SN, Muzzopappa M, Ratcliffe PJ, Wappner P (2002) **Control of the hypoxic response in Drosophila melanogaster by the basic helix-loop-helix PAS protein similar** *Mol Cell Biol* **22**:6842–6853

Manolopoulos KN, Klotz LO, Korsten P, Bornstein SR, Barthel A (2010) **Linking Alzheimer's disease to insulin resistance: the FoxO response to oxidative stress** *Mol Psychiatry* **15**:1046–1052

McAlinden KD, Deshpande DA, Ghavami S, Xenaki D, Sohal SS, Oliver BG, Haghi M, Sharma P (2019) **Autophagy Activation in Asthma Airways Remodeling** *Am J Respir Cell Mol Biol* **60**:541–553

McGuire SE, Mao Z, Davis RL (2004) **Spatiotemporal gene expression targeting with the TARGET and gene-switch systems in Drosophila** *Sci STKE* **2004**

Mikse OR, Blake DC, Jones NR, Sun YW, Amin S, Gallagher CJ, Lazarus P, Weisz J, Herzog CR (2010) **FOXO3 encodes a carcinogen-activated transcription factor frequently deleted in early-stage lung adenocarcinoma** *Cancer Res* **70**:6205–6215

Mori R *et al.* (2014) **Reduced FOXO1 expression accelerates skin wound healing and attenuates scarring** *Am J Pathol* **184**:2465–2479

Orea-Soufi A, Paik J, Braganca J, Donlon TA, Willcox BJ, Link W (2022) **FOXO transcription factors as therapeutic targets in human diseases** *Trends Pharmacol Sci* **43**:1070–1084

Ovrevik J, Lag M, Holme JA, Schwarze PE, Refsnes M (2009) **Cytokine and chemokine expression patterns in lung epithelial cells exposed to components characteristic of particulate air pollution** *Toxicology* **259**:46–53

Pace E, Di Vincenzo S, Ferraro M, Bruno A, Dino P, Bonsignore MR, Battaglia S, Saibene F, Lanata L, Gjomarkaj M (2016) **Carbocysteine counteracts the effects of cigarette smoke on cell growth and on the SIRT1/FoxO3 axis in bronchial epithelial cells** *Exp Gerontol* **81**:119–128

Peng S, Li W, Hou N, Huang N (2020) **A Review of FoxO1-Regulated Metabolic Diseases and Related Drug Discoveries** *Cells*

Pividori M, Schoettler N, Nicolae DL, Ober C, Im HK (2019) **Shared and distinct genetic risk factors for childhood-onset and adult-onset asthma: genome-wide and transcriptome-wide studies** *Lancet Respir Med* **7**:509–522

Pomies P, Blaquiere M, Maury J, Mercier J, Gouzi F, Hayot M (2016) **Involvement of the FoxO1/MuRF1/Atrogin-1 Signaling Pathway in the Oxidative Stress-Induced Atrophy of Cultured Chronic Obstructive Pulmonary Disease Myotubes** *PLoS One* **11**

Potente M, Urbich C, Sasaki K, Hofmann WK, Heeschen C, Aicher A, Kollipara R, DePinho RA, Zeiher AM, Dimmeler S (2005) **Involvement of Foxo transcription factors in angiogenesis and postnatal neovascularization** *J Clin Invest* **115**:2382–2392

Prange R *et al.* (2018) **A Drosophila model of cigarette smoke induced COPD identifies Nrf2 signaling as an expedient target for intervention** *Aging (Albany NY)* **10**:2122–2135

Proud D, Leigh R (2011) **Epithelial cells and airway diseases** *Immunol Rev* **242**:186–204

Raby KL, Michaeloudes C, Tonkin J, Chung KF, Bhavsar PK (2023) **Mechanisms of airway epithelial injury and abnormal repair in asthma and COPD** *Front Immunol* **14**

Raudvere U, Kolberg L, Kuzmin I, Arak T, Adler P, Peterson H, Vilo J (2019) **g:Profiler: a web server for functional enrichment analysis and conversions of gene lists (2019 update)** *Nucleic Acids Res* **47**:W191–W198

Savai R *et al.* (2014) **Pro-proliferative and inflammatory signaling converge on FoxO1 transcription factor in pulmonary hypertension** *Nat Med* **20**:1289–1300

Seiler F *et al.* (2013) **FOXO Transcription Factors Regulate Innate Immune Mechanisms in Respiratory Epithelial Cells** *J Immunol* **190**:1603–1613

Selle J *et al.* (2022) **Maternal and perinatal obesity induce bronchial obstruction and pulmonary hypertension via IL-6-FoxO1-axis in later life** *Nat Commun* **13**

Son HG *et al.* (2018) **Prefoldin 6 mediates longevity response from heat shock factor 1 to FOXO in C. elegans** *Genes Dev* **32**:1562–1575

Spann KM, Baturcam E, Schagen J, Jones C, Straub CP, Preston FM, Chen L, Phipps S, Sly PD, Fantino E (2014) **Viral and host factors determine innate immune responses in airway epithelial cells from children with wheeze and atopy** *Thorax* **69**:918–925

Spreitzer E, Alderson TR, Bourgeois B, Eggenreich L, Habacher H, Brahmersdorfer G, Pritisanac I, Sanchez-Murcia PA, Madl T (2022) **FOXO transcription factors differ in their dynamics and intra/intermolecular interactions** *Curr Res Struct Biol* **4**:118–133

Sun X, Chen WD, Wang YD (2017) **DAF-16/FOXO Transcription Factor in Aging and Longevity** *Front Pharmacol* **8**

Tower J (2011) **Heat shock proteins and Drosophila aging** *Exp Gerontol* **46**:355–362

van der Horst A, Burgering BM (2007) **Stressing the role of FoxO proteins in lifespan and disease** *Nat Rev Mol Cell Biol* **8**:440–450

van der Vos KE, Coffer PJ (2011) **The extending network of FOXO transcriptional target genes** *Antioxid Redox Signal* **14**:579–592

Braga FA Vieira *et al.* (2019) **A cellular census of human lungs identifies novel cell states in health and in asthma** *Nat Med* **25**:1153–1163

von Frieling J, Faisal MN, Sporn F, Pfefferkorn R, Nolte SS, Sommer F, Rosenstiel P, Roeder T (2020) **A high-fat diet induces a microbiota-dependent increase in stem cell activity in the Drosophila intestine** *PLoS Genet* **16**

Wagner C, Isermann K, Fehrenbach H, Roeder T (2008) **Molecular architecture of the fruit fly's airway epithelial immune system** *BMC Genomics* **9**

Wagner C, Isermann K, Roeder T (2009) **Infection induces a survival program and local remodeling in the airway epithelium of the fly** *FASEB J* **23**:2045–2054

Wagner C *et al.* (2021) **Constitutive immune activity promotes JNK-and FoxO-dependent remodeling of Drosophila airways** *Cell Rep* **35**

Wegmann M, Fehrenbach H, Fehrenbach A, Held T, Schramm C, Garn H, Renz H (2005) **Involvement of distal airways in a chronic model of experimental asthma** *Clin Exp Allergy* **35**:1263–1271

Weisberg SP, Ural BB, Farber DL (2021) **Tissue-specific immunity for a changing world** *Cell* **184**:1517–1529

Whitsett JA, Alenghat T (2015) **Respiratory epithelial cells orchestrate pulmonary innate immunity** *Nat Immunol* **16**:27–35

Wolf M, Moser B (2012) **Antimicrobial activities of chemokines: not just a side-effect?** *Front Immunol* **3**

Wu J, He X, Xiong Z, Shi L, Chen D, Feng Y, Wen Q (2022) **Bruceine H Mediates EGFR-TKI Drug Persistence in NSCLC by Notch3-Dependent beta-Catenin Activating FOXO3a Signaling** *Front Oncol* **12**

Xing YQ, Li A, Yang Y, Li XX, Zhang LN, Guo HC (2018) **The regulation of FOXO1 and its role in disease progression** *Life Sci* **193**:124–131

Zepp JA, Morrisey EE (2019) **Cellular crosstalk in the development and regeneration of the respiratory system** *Nature Reviews Molecular Cell Biology* **20**:551–566

Zissler UM *et al.* (2016) **Interleukin-4 and interferon-gamma orchestrate an epithelial polarization in the airways** *Mucosal Immunol* **9**:917–926

Article and author information

Karin Uliczka
Division of Innate Immunity, Priority Research Area Chronic Lung diseases, Research Center Borstel – Leibniz Lung Center, Borstel, Germany, Airway Research Center North (ARCN), Member of the German Center for Lung Research (DZL)

Judith Bossen
Department of Molecular Physiology, Zoology, Kiel University, Kiel, Germany, Airway Research Center North (ARCN), Member of the German Center for Lung Research (DZL)

Ulrich M. Zissler
Center of Allergy and Environment (ZAUM), Technical University Munich and Helmholtz Center Munich, German Research Center for Environmental Health, Munich, Germany, CPC-M, Member of the German Center for Lung Research (DZL)

Christine Fink
Department of Molecular Physiology, Zoology, Kiel University, Kiel, Germany

Xiao Niu
Department of Molecular Physiology, Zoology, Kiel University, Kiel, Germany

Mario Pieper
Anatomical Institute, Lübeck University, Lübeck, Germany

Ruben D. Prange
Department of Molecular Physiology, Zoology, Kiel University, Kiel, Germany

Christina Vock
Division of Experimental Pneumology, Priority Research Area Chronic Lung diseases, Research Center Borstel – Leibniz Lung Center, Borstel, Germany, Airway Research Center North (ARCN), Member of the German Center for Lung Research (DZL)

Christina Wagner
Division of Invertebrate Models, Priority Research Area Chronic Lung diseases, Research Center Borstel – Leibniz Lung Center, Borstel, Germany

Mirjam Knop
Department of Molecular Physiology, Zoology, Kiel University, Kiel, Germany

Ahmed Abdelsadik
Zoology Department, Faculty of Science, Aswan University, 81528, Aswan, Egypt

Sören Franzenburg
IKMB, Kiel University, Kiel, Germany

Iris Bruchhaus
Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany
ORCID iD: 0000-0002-3363-7409

Michael Wegmann
Division of Lung Immunology, Priority Research Area Chronic Lung diseases, Research Center Borstel – Leibniz Lung Center, Borstel, Germany, Airway Research Center North (ARCN), Member of the German Center for Lung Research (DZL)

Carsten B. Schmidt-Weber
Center of Allergy and Environment (ZAUM), Technical University Munich and Helmholtz Center Munich, German Research Center for Environmental Health, Munich, Germany, CPC-M, Member of the German Center for Lung Research (DZL)
ORCID iD: 0000-0002-3203-8084

Peter König
Anatomical Institute, Lübeck University, Lübeck, Germany, Airway Research Center North (ARCN), Member of the German Center for Lung Research (DZL)

Petra Pfefferle
Medical Faculty, University Medical Center Giessen and Marburg, Philipps-Universität Marburg, Marburg, Germany, UGMLC, Member of the German Center for Lung Research (DZL)

Holger Heine
Division of Innate Immunity, Priority Research Area Chronic Lung diseases, Research Center Borstel – Leibniz Lung Center, Borstel, Germany, Airway Research Center North (ARCN), Member of the German Center for Lung Research (DZL)
ORCID iD: 0000-0001-8128-5068

Thomas Roeder
Department of Molecular Physiology, Zoology, Kiel University, Kiel, Germany, Airway Research Center North (ARCN), Member of the German Center for Lung Research (DZL)
For correspondence: troeder@zoologie.uni-kiel.de
ORCID iD: 0000-0002-3489-3834

Copyright

Editors

Reviewing Editor
John Ewer
Universidad de Valparaiso, Valparaiso, Chile

Senior Editor
Pankaj Kapahi
Buck Institute for Research on Aging, Novato, United States of America

Joint Public Review

This work investigates the evolutionary conservation and functional significance of FoxO transcription factors in the response of airway epithelia to diverse stressors, ranging from hypoxia to temperature fluctuations and oxidative stress. Utilizing a comprehensive approach encompassing Drosophila, murine models, and human samples, the study investigates FoxO's role across species. The authors demonstrate that hypoxia triggers a dFOXO-dependent immune response in Drosophila airways, with subsequent nuclear localization of dFOXO in response to various stressors. Transcriptomic analysis reveals differential regulation of crucial gene categories in respiratory tissues, highlighting FoxO's involvement in metabolic pathways, DNA replication, and stress resistance mechanisms.

The study underscores FoxO's importance in maintaining homeostasis by revealing reduced stress resistance in dFOXO Drosophila mutants, shedding light on its protective role against stressors. In mammalian airway cells, FoxO exhibits nuclear translocation in response to hypoxia, accompanied by upregulation of cytokines with antimicrobial activities. Intriguingly, mouse models of asthma show FoxO downregulation, which is also observed in sputum samples from human asthma patients, implicating FoxO dysregulation in respiratory pathologies.

Overall, the manuscript suggests that FoxO signaling plays a critical role in preserving airway epithelial cell homeostasis under stress conditions, with implications for understanding and potentially treating respiratory diseases like asthma. By providing compelling evidence of FoxO's involvement across species and its correlation with disease states, the study underscores the importance of further exploration into FoxO-mediated mechanisms in respiratory health.

Strengths

(1) This study shows that FoxO transcription factors are critical for regulating immune and inflammatory responses across species, and for orchestrating responses to various stressors encountered by airway epithelial cells, including hypoxia, temperature changes, and oxidative stress. Understanding the intricate regulation of FoxO transcription factors provides insights into modulating immune and inflammatory pathways, offering potential avenues for therapeutic interventions against respiratory diseases and other illnesses.

(2) The work employs diverse model systems, including Drosophila, murine models, and human samples, thereby establishing a conserved role for FoxOs in airway epithelium and aiding translational relevance to human health.

(3) The manuscript establishes a strong correlation between FoxO expression levels and respiratory diseases such as asthma. Through analyses of both murine models of asthma and asthmatic human samples, the study demonstrates a consistent reduction in FoxO expression, indicating its potential involvement in the pathogenesis of respiratory disorders. This correlation underscores the clinical relevance of FoxO dysregulation and opens avenues for developing treatments for respiratory conditions like asthma, COPD, and pulmonary fibrosis, addressing significant unmet clinical needs.

(4) The study unveils intriguing mechanistic details regarding FoxO regulation and function. Particularly noteworthy is the observation of distinct regulatory mechanisms governing dFOXO translocation in response to different stressors. The independence of hypoxia-induced dFOXO translocation from JNK signaling adds complexity to our understanding of FoxO-mediated stress responses. Such mechanistic insights deepen our understanding of FoxO biology and pave the way for future investigations into the intricacies of FoxO signaling pathways in airway epithelial cells.

Weaknesses

(1) The manuscript does not distinguish between FoxO expression levels and FoxO activation status. While FoxO nuclear localization is observed in Drosophila and murine models, it remains unclear whether this reflects active FoxO signaling or merely FoxO expression, limiting the mechanistic understanding of FoxO regulation.

(2) The manuscript utilizes various stressors across different experiments without providing a clear rationale for their selection. This lack of coherence in stressor choice complicates the interpretation of results and diminishes the ability to draw meaningful comparisons across experiments.

(3) The manuscript frequently refers to "FoxO signaling" without providing specific signaling readouts. This ambiguity undermines the clarity of the conclusions drawn from the data and hinders the establishment of clear cause-and-effect relationships between FoxO activation and cellular responses to stress.

(4) Many conclusions drawn in the manuscript rely heavily on the quantification of immunostaining images for FoxO nuclear localization. While this is an important observation, it does not provide a sufficient mechanistic understanding of FoxO expression or activation regulation.

(5) The primary weakness in the Drosophila experiments is the analysis of dFoxO in homozygous dFoxO mutant animals, which precludes determining the specific role of dFoxO in airway cells. Despite available tools for tissue-specific gene manipulation, such as tissue-specific RNAi and CRISPR techniques, these approaches were not employed, limiting the precision of the findings.

(6) In mammalian experiments, the results are primarily correlative, lacking causal evidence. While changes in FoxO expression are observed under pathological conditions, the absence of experiments on FoxO-deficient cells or tissues precludes establishing a causal relationship between FoxO dysregulation and respiratory pathologies.

(7) Although the evidence suggests a critical role for FoxO in airway tissues, the precise nature of this role remains unclear. With gene expression changes analyzed only in Drosophila, the extent of conservation in downstream FoxO-mediated pathways between mammals and Drosophila remains uncertain. Additionally, the functional consequences of FoxO deficiency in airway cells were not determined, hindering comparisons between species and limiting insights into FoxO's functional roles in different contexts.

https://doi.org/10.7554/eLife.96385.1.sa0