Regulatory control of epithelial damage during *Aspergillus fumigatus* infection

S R Khan[1]*, N Van Rhijn[1] ,Z Carter[1], L Gregson[1], D Thomson[1], P Papastamoulis[1], M Rattray[1], M Bromley[1], E Bignell[1]

[1] School of Biological Sciences, University of Manchester, UK

1. Introduction

- A unifying feature of *Aspergillus fumigatus* lung disease is epithelial damage, the regulatory control and the mechanistic basis of which is poorly characterised.
- Previous studies[1] have identified a number of fungal attributes such as epithelial adhesion, spore uptake, germination, hyphal penetration and release of secreted factors as critical contributors to epithelial damage during *A. fumigatus* invasive infection.
- This study aims to identify, characterise and mechanistically understand the *A. fumigatus* transcription factors driving those activities during epithelial infection.

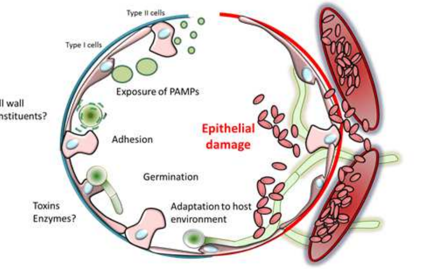


Figure 1: Schematic of interaction of *A. fumigatus* inside the alveoli of a lung. *A. fumigatus* conidia initiate pathogenesis by a series of events leading to epithelial disintegration causing invasive disease.

2. High-throughput screening for epithelial damage

In order to identify the regulators of epithelial cell damage, an A549 epithelial cell model was challenged with 479 *A. fumigatus* transcription factor mutant strains and epithelial damage was assessed by screening for cell detachment and cell lysis.

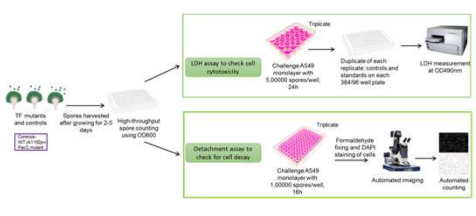
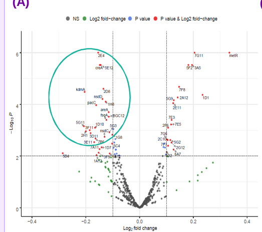

TF mutants and controls
Spores harvested after growing for 2-5 days
High-throughput spore counting using OD600
LDH assay to check cell cytotoxicity
Triplicate
Challenge A549 monolayer with 5,00000 spores/well, 24h
Duplicate of each replicate: controls and standards on each 384/96 well plate
LDH measurement at OD490nm
Detachment assay to check for cell decay
Challenge A549 monolayer with 1,00000 spores/well, 16h
Formaldehyde fixing and DAPI staining of cells
Automated imaging
Automated counting

Figure 2: High-throughput methodologies used for screening epithelial damage. The TF mutant library was grown for 2-5 days to harvest sufficient spores; the spores were counted using OD[600] measurement and were challenged against A549 cells. LDH in cell supernatant was measured by OD[490] after 24 hours in a 96 or 384 well plate for quantitation of cell lysis. Detachment of epithelial cell monolayer after 16 hours was imaged and quantitated automatedly in a 96 well plate to determine cell decay.

3. Regulators of epithelial damage

3.1 Epithelial cell detachment

(A)



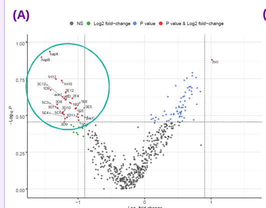
(B)

TF number	Gene ID	Generic name	Main Function	References
2 E4	AFUB_046160	Not characterised	Unknown	~
1G11	AFUB_027530	CreA	Hypoxic adaptation, carbon catabolite repression	Beattie *et al*. 2017
5 E12	AFUB_019830	Not characterised	Unknown	~
1C3	AFUB_011800	KdmA	Secondary metabolism in *A.nidulans*	Gacek-Matthews *et al*. 2015
2D8	AFUB_044930	Not characterised	Unknown	~
2A7	AFUB_035330	NsdD	Early stage of mating,resistance toward cell wall stress	Szewczyk & Krappmann. 2010
2B5	AFUB_037210	PacC	pH response, epithelial entry and invasion, protease production	Bertuzzi *et al*. 2014; Bignell *et al*. 2004
1H8	AFUB_031000	Not characterised	Unknown	~
7G8	AFUB_096370	AreA	Nitrogen utilization	S. Krappmann & Braus, 2005
5 E1	AFUB_094860	FsqA	Secondary metabolite production	Elaine *et al*. 2016
2 A3	AFUB_033930	BGC12	Secondary metabolite production	Elaine *et al*. 2017
5G11	AFUB_077270	Not characterised	Unknown	~
1D10	AFUB_015560	Not characterised	Unknown	~
3F11	AFUB_082950	Not characterised	Unknown	~
5G3	AFUB_064180	Not characterised	Unknown	~
5H5	AFUB_089440	NsdC	Early stage of mating, resistance toward cell wall stress	Szewczyk & Krappmann. 2010
5C4	AFUB_047970	Not characterised	Unknown	~
1G8	AFUB_026210	Not characterised	Unknown	~
2H1	AFUB_056620	Not characterised	Unknown	~
3D11	AFUB_077460	Not characterised	Unknown	~
3 E11	AFUB_079860	Not characterised	Unknown	~
7B6	AFUB_025730	NdtA	Nutrient stress in A.nidulans	~
7A11	AFUB_017960	Not characterised	Unknown	~
1D7	AFUB_015090	Not characterised	Unknown	~
1A12	AFUB_005510	Not characterised	Unknown	~
3F9	AFUB_082080	Not characterised	Unknown	~
6A12	AFUB_042180	Not characterised	Unknown	~
5B4	AFUB_023530	Not characterised	Unknown	~

Figure 4: Screening for regulators of epithelial cell detachment. (A) Volcano plot showing the output from the detachment screen transformed as fold change number of adherent cells for the 479 *A. fumigatus* TF mutants, with biological difference in the x axis and statistical difference in the y axis. **B)** Table showing the identified TFs driving epithelial cell detachment.

3.2 Epithelial cell lysis

(A)



(B)

TF number	Gene ID	Generic name	Main Function	References
2G3	AFUB_052420	HapX	Iron homeostasis (deplete conditions)	Gsaller *et al*. 2014; Schrettl et al.2010
1H5	AFUB_030360	HapB	Iron homeostasis (deplete conditions)	Gsaller *et al*. 2014; Schrettl *et al*.2011
1H12	AFUB_033470	Not characterised	Unknown	~
1H10	AFUB_031980	Not characterised	Unknown	~
3C12	AFUB_074510	Not characterised	Unknown	~
1D9	AFUB_015440	Not characterised	Unknown	~
2 E12	AFUB_048380	Not characterised	Unknown	~
2C7	AFUB_041100	Ace1	Conidial formation, cell wall architecture	Ejzykowicz *et al*. 2009
3 E3	AFUB_078150	AtfD	Stress response to conidia	Hagiwara, Suzuki, Kamei, Gonoi, & Kawamoto. 2014
2F4	AFUB_049550	Not characterised	Unknown	~
3D8	AFUB_076200	Not characterised	Unknown	~
5C3	AFUB_046750	Not characterised	Unknown	~
3D11	AFUB_077460	Not characterised	Unknown	~
3D10	AFUB_077250	Not characterised	Unknown	~
5 E4	AFUB_099770	Not characterised	Unknown	~
5C5	AFUB_048230	Not characterised	Unknown	~
3D9	AFUB_077130	Not characterised	Unknown	~
1B2	AFUB_006120	Not characterised	Unknown	~
3 E5	AFUB_078520	Not characterised	Unknown	~
2B5	AFUB_037210	PacC	pH response, epithelial entry and invasion, protease production	Bertuzzi *et al*. 2014; Bignell *et al*. 2004
1D11	AFUB_015750	Not characterised	Unknown	~
1 E6	AFUB_016720	Not characterised	Unknown	~
1 E5	AFUB_016630	Not characterised	Unknown	~
3D1	AFUB_074560	Not characterised	Unknown	~

Figure 5: Screening for regulators of epithelial cell lysis. A) Volcano plot showing the output from the LDH screen for the 479 *A. fumigatus* TF mutants, with confidence interval from bio-informatic analyses on the y axis versus fold change LDH to the WT on the x axis **B)** Table showing the identified TFs driving epithelial cell lysis.

4. Epithelial damage and growth

Screening of the 479 *A. fumigatus* TF mutants for growth using OD measurement at 24 hours identified the slow growing TF mutants.

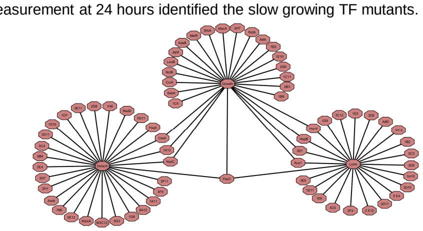


Figure 6: Correlation of *A. fumigatus* regulators of epithelial damage with growth. *A. fumigatus* TFs driving epithelial cell detachment (in left) and epithelial cell lysis (in right) and their relation with slow growing mutants (in centre).

5. Attributes driving epithelial damage

In order to identify factors driving epithelial damage by the identified regulators of epithelial damage, the TF mutants were tested for attributes previously reported to contribute to epithelial damage[1].

5.1 Adhesion capacity to epithelial cells

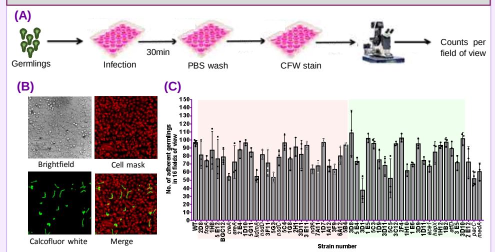


Figure 7: Adhesion capacity of *A. fumigatus* mutants to A549 cells. (A) Germlings incubated with A549 cells for 30 min to enable adhesion were washed to stain and visualise only the adherent germlings **(B)** Representative images of brightfield, cell mask stained A549 cells and calcofluor white stained germlings captured with TCS-SP8 confocal microscope at 40x magnification **(C)** Total number of adherent germlings for the identified *A. fumigatus* TF mutants. WT=parental strain

5.2 Germination efficiency and hyphal extension

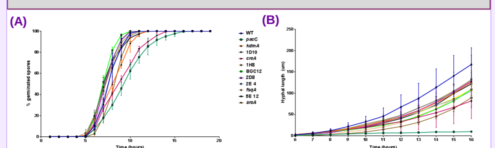


Figure 8: Germination efficiency and hyphal lengths of *A. fumigatus* mutants. (A) Percentage germinated spores and (B) hyphal lengths of the identified TF mutants calculated by assessing growth using time-lapse imaging with the TCS-SP8 confocal microscope. WT=parental strain

5.3 Spore internalisation by epithelial cells

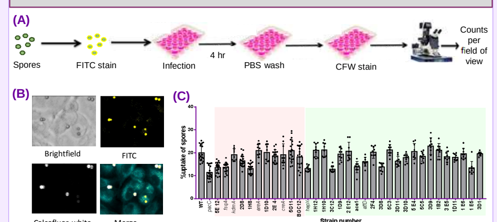


Figure 9: Internalisation of *A. fumigatus* mutant spores by A549 cells. (A) Spores incubated with A549 cells for 4 hours to enable internalisation were washed to stain and visualise the internal and external spores **(B)** Representative images of brightfield, FITC (all spores), calcofluor white (external adherent spores) captured using the TCS-SP8 confocal microscope at 40x magnification **(C)** Percentage spore uptake for the identified *A. fumigatus* TF mutants. WT=parental strain

5.4 Cell wall defects

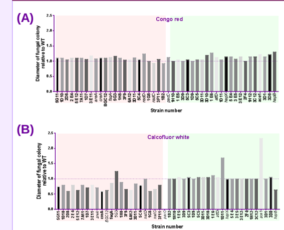


Figure 10: Sensitivity of *A. fumigatus* mutants to cell wall destabilising agents. Cell wall defects in the identified TF mutants were screened using colony diameter measurement of 10^3 spores after 48 hours of growth on solid media containing cell wall destabilising agents. **(A)** Diameter of fungal colony on media containing Congo red relative to the WT **(B)** Diameter of fungal colony on media containing calcofluor white relative to the WT. WT=parental strain

5.5 Secreted factors

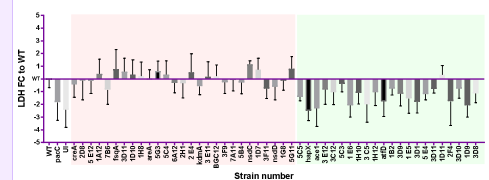


Figure 11: Damage caused by secreted factors of *A. fumigatus* mutants to epithelial cells. Culture filtrate prepared by growing the TF mutants for 48 hours were challenged with A549 cells and LDH released on infection with the culture filtrate was measured to determine the effect the secreted factors on epithelial damage. WT=parental strain

6. Summary

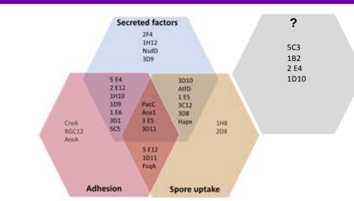


Figure 12: Attributes of the TF mutants driving epithelial damage. Summary Venn diagram showing overlap for attributes of adhesion capacity, spore uptake and secreted factor damage for 33 of the regulators of epithelial damage.

7. Conclusion and Future work

- This study for the first time has identified and characterised the transcription factors regulating epithelial damage during *A. fumigatus* infection.
- Each of the transcription factor mutants exhibited a unique phenotypic profile with respect to epithelial adhesion, cell wall defects, and uptake of spores by epithelial cells.
- Future work will involve systems biology approaches to map the architecture of the regulatory network driving epithelial damage during *A. fumigatus* infection.

Faculty of Biology, Medicine and Health

REFERENCES: [1] Bertuzzi, M., Icheoku, U., van Rhijn, N., Hayes, G., Denning, D., Osherov, N., & Bignell, E.(2018). Anti-Aspergillus Activities of the Respiratory Epithelium in Health and Disease. *Journal of Fungi*, *4*(1), 8.