

Human respiratory syncytial virus non-structural protein NS1 modifies miR-24 expression via transforming growth factor- β

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Human respiratory syncytial virus (RSV) is a major health challenge in the young and elderly owing to the lack of a safe and effective vaccine and proven antiviral drugs. Understanding the mechanisms by which viral genes and proteins modulate the host response to infection is critical for identifying novel disease intervention strategies. In this study, the RSV non-structural protein NS1 was shown to suppress miR-24 expression during infection. Lack of NS1 was linked to increased expression of miR-24, whilst NS1 overexpression suppressed miR-24 expression. NS1 was found to induce Kruppel-like factor 6 (KLF6), a transcription factor that positively regulates the transforming growth factor (TGF)- β pathway to induce cell cycle arrest. Silencing of KLF6 led to increased miR-24 expression via downregulation of TGF- β . Treatment with exogenous TGF- β suppressed miR-24 expression and induced KLF6. Confocal microscopy showed co-localization of KLF6 and RSV NS1. These findings indicated that RSV NS1 interacts with KLF6 and modulates miR-24 expression and TGF- β , which facilitates RSV replication.

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INTRODUCTION

Human respiratory syncytial virus (RSV) is a ubiquitous negative-sense ssRNA virus that can cause severe lung disease following infection (CDC, 2013; Hall *et al.*, 2009; Nair *et al.*, 2010). RSV is a member of the genus *Pneumovirus*, family *Paramyxoviridae* with a non-segmented genome that encodes 10 genes and 11 proteins (NS1, NS2, N, P, M, SH, G, F, M2-1, M2-2 and L). The two non-structural proteins (NS1 and NS2), which are not part of the intact virion, are transcribed and translated during infection (Moore *et al.*, 2008).

RSV infection rates are high and by age 2 years the majority of young children have experienced at least one infection (CDC, 2008, 2013; Hall *et al.*, 2009; Mori *et al.*, 2014; Nair *et al.*, 2010; Stockman *et al.*, 2012; Zhou *et al.*, 2012). RSV infection in high-risk individuals, such as infants, young children, immunocompromised adults and the elderly, can manifest as serious pulmonary inflammatory disease including bronchiolitis and pneumonia (Hoffman *et al.*, 2004; Moore *et al.*, 2013; Openshaw & Chiu, 2013; Oshansky

et al., 2009b; Psarras *et al.*, 2004; Vicencio, 2010). There is also substantial evidence that early RSV infection can mediate airway remodelling, a feature that predisposes individuals to asthma development and exacerbation (Fong *et al.*, 2000; Foronjy *et al.*, 2014; Hirakawa *et al.*, 2013; Hotard *et al.*, 2015; Liesman *et al.*, 2014; Meng *et al.*, 2014; Piedimonte, 2002, 2003; Tan *et al.*, 2008; Wu *et al.*, 2011). Transforming growth factor (TGF)- β is expressed during RSV infection of lung epithelial cells (Gibbs *et al.*, 2009; McCann & Imani, 2007; Mgbemena *et al.*, 2011) and several studies indicate that it plays an important role in asthma development (de Faria *et al.*, 2008; Fong *et al.*, 2000; Gagliardo *et al.*, 2013; Hoshino *et al.*, 1998; Howell & McAnulty, 2006; Pelaia *et al.*, 2007; Sharma *et al.*, 2009). TGF- β also regulates aspects of the inflammatory cytokine response to RSV infection (Thornburg *et al.*, 2010) and cell cycle arrest (Gibbs *et al.*, 2009; McCann & Imani, 2007; Wu *et al.*, 2011), although the mechanism(s) are not completely known. Kruppel-like factor 6 (KLF6), an evolutionarily conserved and ubiquitously expressed zinc-finger protein that belongs to the mammalian Sp1/KLF family of transcriptional regulators (Andreoli *et al.*, 2010), is a critical transcription factor required for TGF- β gene expression during RSV infection of human lung epithelial cells (Mgbemena *et al.*, 2011).

One supplementary table and three supplementary figures are available with the online Supplementary Material.

RSV has a tropism for respiratory epithelial cells, thus human type II lung epithelial (A549) cells are often used to model the host response to RSV infection (Gibbs *et al.*, 2009; Martínez *et al.*, 2007; Munday *et al.*, 2010). RSV attaches to cells by its two major surface proteins, the attachment (G) and fusion (F) proteins, which bind to cellular glycosaminoglycans (Hallak *et al.*, 2000). RSV F and G proteins also bind to several other cell constituents, such as CX3CR1 molecules (Tripp *et al.*, 2001), Toll-like receptor 4 (TLR4) (Kurt-Jones *et al.*, 2000), RhoA (Budge & Graham, 2004; Pastey *et al.*, 1999), intercellular adhesion molecule 1 (Mastrangelo & Hegele, 2013; Mata *et al.*, 2012) and nucleolin (Tayyari *et al.*, 2011). These interactions may facilitate infection and contribute to tropism.

The virus–host interactions that follow RSV binding are modified and regulated by several RSV proteins, including non-structural and G proteins, which modulate pro-inflammatory and antiviral cytokine expression by host cells (Atreya *et al.*, 1998; Bossert *et al.*, 2003; Boyapalle *et al.*, 2012; Elliott *et al.*, 2007; Kotelkin *et al.*, 2006; Liesman *et al.*, 2014; Ling *et al.*, 2009; Lo *et al.*, 2005; Moore *et al.*, 2008; Munir *et al.*, 2011; Ren *et al.*, 2011; Schlender *et al.*, 2000; Spann *et al.*, 2004, 2005; Wright *et al.*, 2006; Wu *et al.*, 2012; Xu *et al.*, 2014). RSV non-structural proteins inhibit minigenome transcription and viral RNA replication (Atreya *et al.*, 1998), and abrogate the innate host response to infection in part by controlling expression of host cell proteins involved in the cell cycle and replication (Atreya *et al.*, 1998; Liesman *et al.*, 2014; Wu *et al.*, 2012). NS2 antagonizes IFN- β activation by binding to retinoic acid-inducible gene 1 (Ling *et al.*, 2009), and NS1 and NS2 inhibit activation of IFN regulatory factor 3 (IRF3) (Bossert *et al.*, 2003; Spann *et al.*, 2005), promoting degradation of transcription factors for SOCS (suppressor of cytokine signalling) proteins (Moore *et al.*, 2008; Xu *et al.*, 2014). RSV lacking NS1 and NS2 genes are attenuated *in vivo* (Jin *et al.*, 2000; Kong *et al.*, 2007; Luongo *et al.*, 2013; Straub *et al.*, 2011; Teng & Collins, 1999; Teng *et al.*, 2000; Whitehead *et al.*, 1999). Normal human bronchial epithelial (NHBE) cells overexpressing RSV NS1 show reduced HLA-DR, CD80 and CD86 expression, and inhibition of T-helper T_h1, T_h2 and T_h17 cell differentiation, whilst overexpression of RSV NS2 inhibits T_h2 and T_h17 cell differentiation (Qin *et al.*, 2014). Codon deoptimized RSV is attenuated *in vitro*, but evokes a strong humoral response upon vaccination and challenge (Meng *et al.*, 2014). These and other immune modulators inhibit the development of the antiviral state (Bossert *et al.*, 2003; Elliott *et al.*, 2007; Goswami *et al.*, 2013; Lo *et al.*, 2005; Moore *et al.*, 2008; Oshansky *et al.*, 2009a; Spann *et al.*, 2005).

The role of microRNAs (miRNAs) in post-transcriptional regulation of host genes responding to RSV infection is not fully understood. miRNA expression is known to be regulated by multiple processes including TLR4 signalling (Nahid *et al.*, 2009, 2011a, b; O'Connell *et al.*, 2007; Pauley *et al.*, 2010; Schulte *et al.*, 2013; Taganov *et al.*, 2006), IRF3 activation (Liu *et al.*, 2009), IFN-stimulated

gene transcripts (Eis *et al.*, 2005) and by viral proteins such as RSV G (Bakre *et al.*, 2012). RSV infection of A549 cells affects a set of miRNAs (five that are induced and two which are repressed), particularly *let-7f* expression, which is induced primarily by RSV G (Bakre *et al.*, 2012). Treatment of A549 cells with purified RSV G enhanced *let-7f* expression, a feature not observed following RSV F treatment. Importantly, modulation of the *let-7* family of miRNAs with miRNA mimics and inhibitors affected RSV replication, indicating that RSV modulates host miRNA expression to affect the outcome of the antiviral host response and this is mediated to a considerable extent by RSV G protein expression (Bakre *et al.*, 2012).

The data presented here, and that from related research (Thornburg *et al.*, 2012), suggest that RSV NS1 suppresses host miRNA expression. In this study, it is shown that recombinant RSV lacking the NS1 gene induces miR-24 expression, and others have shown that ablation of NS1 and NS2 results in elevated *let-7i* and miR-30b expression (Thornburg *et al.*, 2012). The molecular pathways by which RSV NS1 modulates miRNA expression have been fully elucidated, although the findings demonstrate a role for TGF- β in miR-24 suppression. Specifically, in this study it is shown that the NS1 induces transcription factor KLF6, a positive regulator of TGF- β (Mgbemena *et al.*, 2011), which then suppresses miR-24 in a feed-forward pathway that further induces TGF- β . These findings demonstrate a novel mechanism in which RSV NS1 potentially interacts with KLF6 to modulate miR-24 and TGF- β , thus facilitating cell cycle arrest, reduced apoptosis and elevated RSV replication (Bakre *et al.*, 2012; Gibbs *et al.*, 2009; Mgbemena *et al.*, 2011).

RESULTS

RSV NS1 suppresses miR-24 expression

To investigate the role of RSV non-structural proteins in modulating miR-24 expression, A549 cells were infected (m.o.i. 1.0) with sucrose-purified (sp) or unpurified WT (RSV A2) or recombinant RSV A2 lacking NS1 [rA2 Δ NS1EGFP (Δ NS1)] or NS2 [rA2 Δ NS2EGFP (Δ NS2)] genes. miR-24 expression was determined relative to 18S rRNA as described previously (Bakre *et al.*, 2012). miR-24 expression was induced \sim 20-fold at 24 h with RSV A2 infection and was further increased in Δ NS1 infection (Fig. 1a). Similar data were obtained for sucrose-purified and unpurified viruses. To confirm if this was modulated by NS1 and/or NS2, A549 cells were transfected with plasmids expressing RSV NS1/NS2 fused to EGFP or control plasmid expressing EGFP alone. NS1 and NS2 expression was validated by fluorescence microscopy beginning 6 h post-transfection (Fig. S1, available in the online Supplementary Material). miR-24 expression was suppressed by NS1 (but less so by NS2) overexpression at all time points tested, indicating that RSV NS1 suppressed miR-24 expression. Plaque titres showed equivalent replication

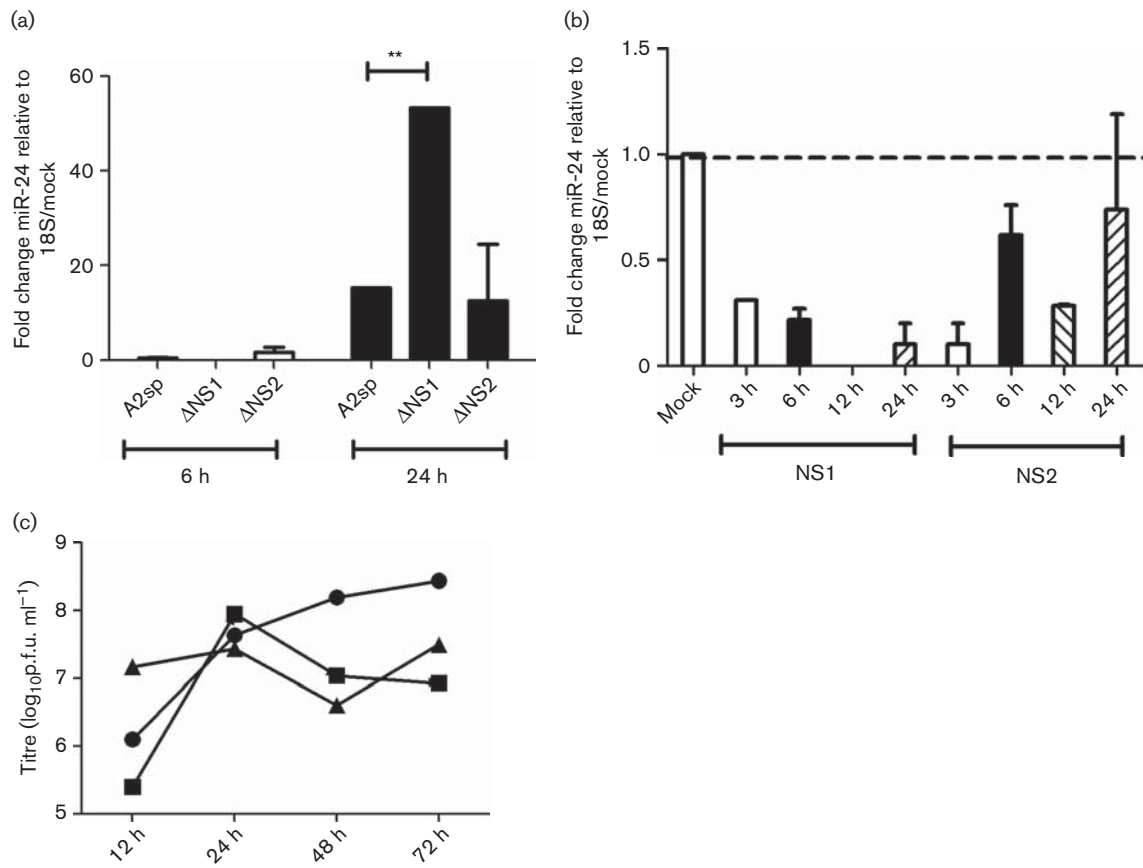


Fig. 1. RSV NS1 modulates miR-24 expression. (a) Lack of RSV NS1 induces miR-24 expression. A549 cells were infected with sucrose-purified (sp) RSV A2 (A2sp), Δ NS1 or Δ NS2 virus (m.o.i. 1.0) for 6 or 24 h followed by RNA isolation and quantitative real-time (qRT)-PCR for miR-24 relative to A549 cells treated with 30% sucrose as mock and 18S rRNA as a housekeeping control. (b) RSV NS1 overexpression suppresses miR-24. miR-24 expression was measured relative to 18S rRNA in A549 cells transfected with pEGFPC1-NS1 or pEGFPC1-NS2 (50 ng each) plasmids for varying times. Data shown are representative of three independent experiments. (c) WT and Δ NS1/ Δ NS2 viruses grow at equivalent titres at early time points. Viral titres of RSV A2 (●), Δ NS1 (■) or Δ NS2 (▲) viruses were determined by plating serial 10-fold dilutions of infected A549 lysates on Vero E6 cells as per the standard protocol and visualized by staining for RSV F protein using mAb-131-2A. Data represent mean \pm SEM of four biological replicates. ** $P < 0.01$.

of RSV A2, Δ NS1 and Δ NS2 to 24 h (Fig. 1c). RSV Δ NS1 and Δ NS2 viruses exhibited delayed growth 24 h post-infection (p.i.) due to an inability to counter the host innate immune response, as has been reported previously (Spann *et al.*, 2005). As the opposite phenotypes occurred with NS1 deficiency versus overexpression, these data suggested that NS1 was the predominant regulator of miRNA expression and subsequent experiments were focused on elucidating this role.

RSV NS1 modulates TGF- β expression via KLF6

RSV non-structural proteins have been shown to interact with several host nuclear, cytosolic and mitochondrial factors, leading to suppression of the antiviral response, cell cycle regulation, DNA damage repair and culminating

in G_0/G_1 phase cell cycle arrest (Atreya *et al.*, 1998; Bossert *et al.*, 2003; Boyapalle *et al.*, 2012; Elliott *et al.*, 2007; Lo *et al.*, 2005; Munday *et al.*, 2010; Munir *et al.*, 2011; Ren *et al.*, 2011; Schlender *et al.*, 2000; Spann *et al.*, 2004, 2005; Wu *et al.*, 2012; Xu *et al.*, 2014). TGF- β expression during RSV infection has been shown to be important for cell cycle inhibition in the G_0/G_1 phase (Gibbs *et al.*, 2009; McCann & Imani, 2007; Wu *et al.*, 2011) and miR-24 has been shown to be suppressed by TGF- β (Sun *et al.*, 2008), as well as regulate TGF- β precursor processing (Luna *et al.*, 2011). As preliminary data showed a role for NS1 in regulating miR-24 expression, the impact of NS1 overexpression on TGF- β expression was determined. The TGF- β transcript was significantly ($P < 0.05$) induced post-NS1 overexpression (Fig. 2a). This finding is in agreement with a previous study showing TGF- β was

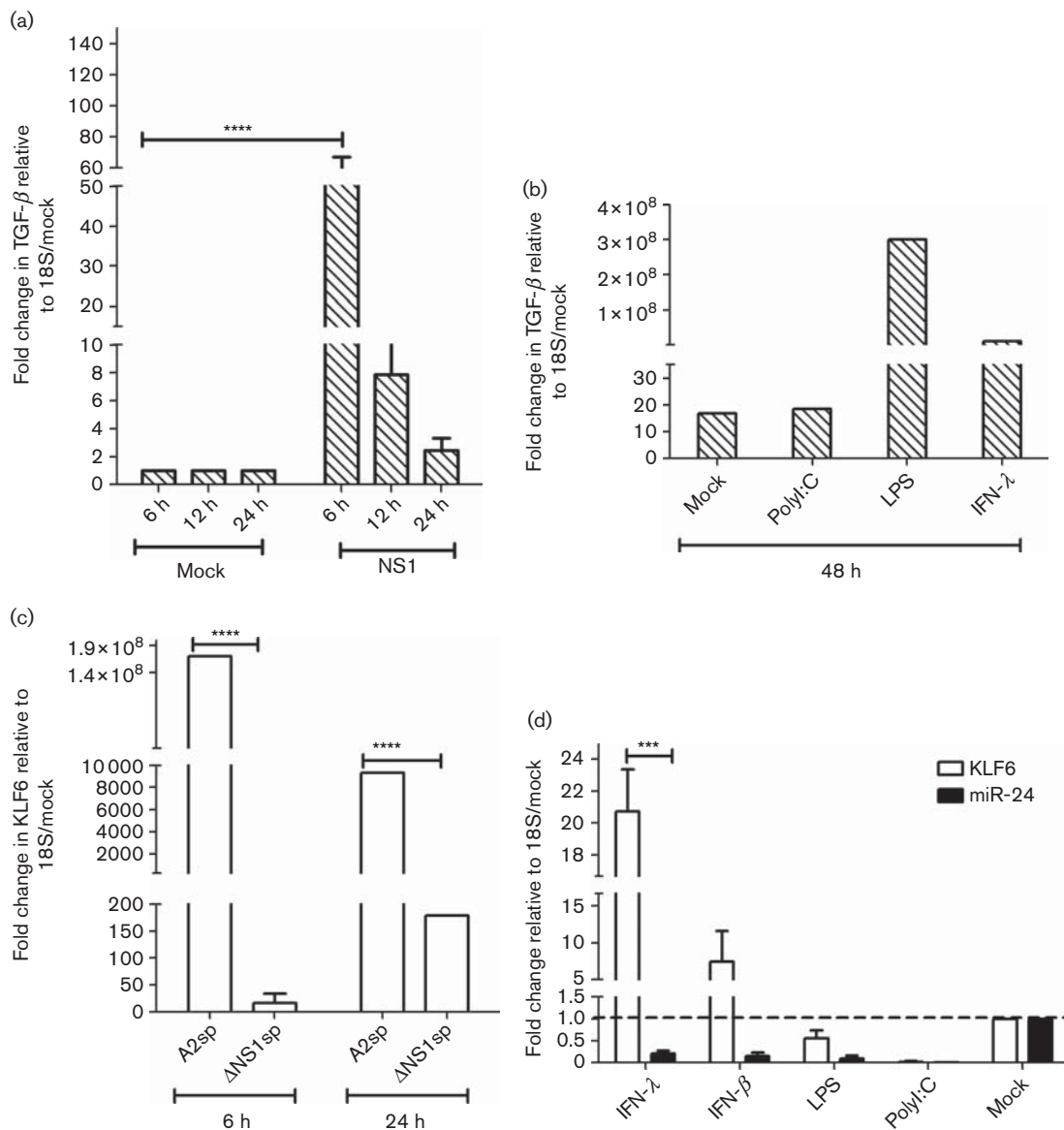


Fig. 2. RSV NS1 and IFN- λ induce TGF- β expression via KLF6. (a) Overexpression of RSV NS1 induces TGF- β . A549 cells were transfected with pEGFPC1-NS1 or pEGFPC1 alone for varying times and TGF- β expression was determined by qRT-PCR at different time points following NS1 overexpression. Data represent mean \pm SEM of three independent experiments. (b) Secondary triggers of TGF- β expression. NS1-transfected A549 cells were either mock treated, or treated with recombinant IFN- λ (10 ng ml^{-1}) or LPS ($1 \mu\text{g ml}^{-1}$), or transfected with high-molecular-mass polyI:C (10 ng ml^{-1}) for 24 h. TGF- β expression was determined by qRT-PCR. (c) RSV NS1 induces KLF6 expression. WT RSV A2 but not Δ NS1 infection induces KLF6 expression. A549 cells were infected with sucrose purified (sp) WT RSV A2 or recombinant Δ NS1 virus (m.o.i. 1.0) for 24 h followed by qRT-PCR analysis of KLF6 expression. Data represent two independent experiments. (d) Secondary triggers of KLF6 expression. pEGFPC1-NS1-transfected A549 cells were mock treated, or treated with IFN- λ (10 ng ml^{-1}), IFN- β (10 ng ml^{-1}) or LPS ($1 \mu\text{g ml}^{-1}$) for 24 h in two independent experiments. KLF6 or miR-24 expression was measured relative to 18S rRNA. *** $P < 0.001$; **** $P < 0.0001$.

induced following RSV infection and caused cell cycle arrest (Gibbs *et al.*, 2009). Additionally, as early as 6 h post-treatment, it was shown that treatment of NS1-transfected cells with recombinant IFN- λ and lipopolysaccharide (LPS) but not polyI:C or IFN- β

expression (Fig. 2b). As type III IFNs constitute the majority of antiviral cytokines produced during RSV infection (both at early and late time points), these data suggest that type III IFNs expressed by RSV-infected cells may contribute to cell cycle arrest via induction of

TGF- β in neighbouring uninfected and infected cells, facilitating viral replication and persistence.

Transcription factor KLF6 positively regulates the expression of TGF- β during RSV infection by binding to the TGF- β promoter and activating TGF- β transcription (Mgbemena *et al.*, 2011). Silencing KLF6 abrogates TGF- β expression and reduces RSV replication (Mgbemena *et al.*, 2011). KLF6 expression was found to be significantly ($P < 0.05$) increased in cells infected with RSV, but not with Δ NS1 RSV at 6 h p.i. At 24 h p.i., KLF6 was induced in Δ NS1-infected cells, but was significantly lower ($P < 0.05$) relative to WT infection, suggesting that NS1 is a major, but not the only, driver of KLF6 induction (Fig. 2c). To determine if KLF6 expression was IFN dependent or independent, cells were treated with LPS, polyI:C, or types I or III IFN, and it was found that treatment with exogenous IFN- λ and IFN- β , but not LPS or polyI:C, significantly ($P < 0.05$) induced KLF6, but not miR-24 expression (Fig. 2d). The substantial increase in TGF- β upon NS1 overexpression and increase in KLF6 expression in WT versus Δ NS1 infection supported the hypothesis that NS1 positively regulated TGF- β expression via induction of KLF6. Additionally, induction of KLF6 by IFN- λ /IFN- β but not LPS/polyI:C suggested that these cytokines could induce KLF6 expression via the Jak-Stat (Janus kinase-signal transducer and activator of transcription) pathway. Treatment with IFN- λ does cause cell cycle arrest at G₀/G₁ phase in A549 (Li *et al.*, 2012) as well as other cell lines (Li *et al.*, 2008, 2010).

Silencing KLF6 induces miR-24 and TGF- β treatment represses miR-24 expression

As KLF6 has been shown to induce TGF- β during RSV infection (Mgbemena *et al.*, 2011) and TGF- β has been shown to suppress miR-24 expression, the effect of KLF6 gene silencing on miR-24 expression was examined. A549 cells were transfected with small interfering RNA (siRNA) SMARTpools (four different siRNAs per gene) targeting KLF6 (siKLF6) to knockdown KLF6 expression and subsequently infected with RSV. Expression levels of miR-24, RSV M2 and TGF- β were determined 24 h post-treatment using quantitative real-time qRT-PCR relative to mock-transfected cells. siKLF6 SMARTpools knocked down KLF6 mRNA (data not shown), led to decreased TGF- β and also reduced viral replication (Fig. 3a). In contrast, miR-24 expression was considerably upregulated, supporting the hypothesis that NS1-induced TGF- β suppresses miR-24 expression and removing KLF6, the positive inducer of TGF- β , relieves TGF- β -mediated miR-24 suppression (Fig. 3a).

TGF- β treatment has been shown to increase RSV replication in A549 as well as primary NHBE cells (Gibbs *et al.*, 2009). TGF- β has also been shown to inhibit miR-24 expression in other cell types (Cao *et al.*, 2012; Sun *et al.*, 2008) via Smad3 transcription factor. To validate the KLF6 silencing observations, cells were treated with recombinant TGF- β for various times (1, 6, 12 and 24 h), and expression of KLF6 and miR-24 determined by qRT-PCR. Treatment with recombinant TGF- β 1 led to a rapid

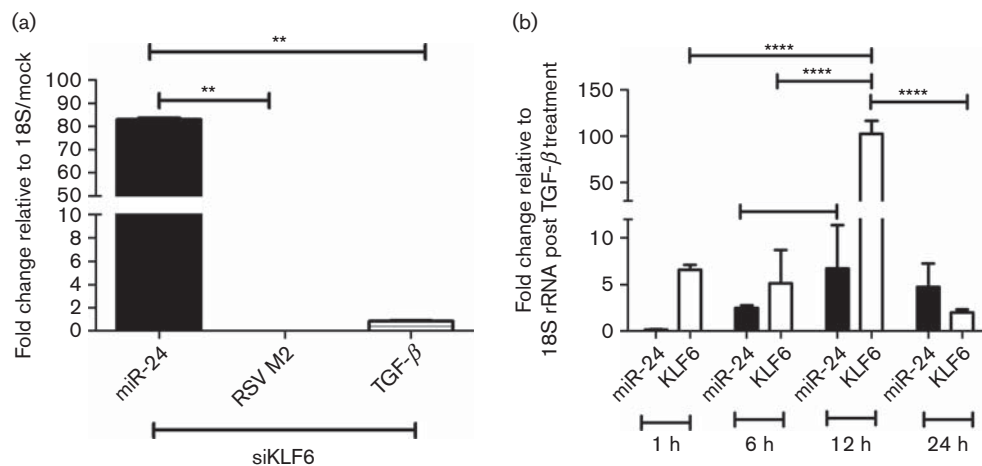


Fig. 3. KLF6 suppresses miR-24 via TGF- β . (a) KLF6 silencing induces miR-24, and represses TGF- β expression and viral replication. A549 cells were either mock treated or transfected with 25 nM siRNA SMARTpools against KLF6 in DharmaFECT 1 for 24 h. Plates with similar treatment were infected with RSV A2 (m.o.i. 1.0). Total RNA was isolated 24 h post-transfection using RNAzol RT and used to measure miR-24, RSV M2 and TGF- β expression relative to 18S rRNA. Data represent mean \pm SEM from two independent experiments. (b) Recombinant TGF- β suppresses miR-24 and induces KLF6. A549 cells were treated with recombinant human TGF- β (10 ng ml⁻¹) for 6, 12 and 24 h. Total RNA was isolated at the respective time points using RNAzol RT, and analysed for miR-24 and KLF6 expression relative to 18S rRNA. Data represent mean \pm SEM from three independent experiments. ** $P < 0.01$, **** $P < 0.0001$.

induction of KLF6 expression which peaked at 12 h, whilst miR-24 levels increased by twofold and remained unchanged at 24 h (Fig. 3b).

miR-24 repression during infection modulates multiple cellular pathways

Inhibition of miR-24 in A549 cells has been shown to prevent cell cycle progression (Cheng *et al.*, 2005). To determine the broader impact of miR-24 repression during RSV infection, potential miR-24 targets were short-listed by comparing the top 500 predicted miR-24 targets based on their P_{CT} (probability of conserved targeting) scores (Friedman *et al.*, 2009; Garcia *et al.*, 2011; Grimson *et al.*, 2007; Lewis *et al.*, 2005) with those deregulated during RSV infection (Fig. 4a, b). An overlapping set of genes was generated which was further categorized into functional clusters using DAVID (Huang *et al.*, 2009). Clusters with enrichment scores ≥ 1.3 (corresponding to non-log $P \leq 0.05$) were considered significant and 10 target genes [Pim-1 proto-oncogene-1 (PIM1), polo-like kinase 3 (PLK3), dual-specificity tyrosine phosphorylation-regulated

kinase 2 (DYRK2), serine threonine kinase 10 (STK10), cyclin-dependent kinase inhibitor 1 (CDKN1A), Nemo-like kinase (NLK), casein kinase $\gamma 1$ (CSNK1G1), fibroblast growth factor 3 (FGFR3), platelet-derived growth factor β (PDGFRB) and KLF6 that met the above criteria were chosen for validation for miR-24 regulation using a miR-24 inhibition assay (Fig. 4c). It was hypothesized that miR-24 inhibitor would relieve miR-24 repression of genuine target genes relative to mock-transfected or negative controls as measured by qRT-PCR. A549 cells were transfected with miR-24 inhibitors, or negative controls, for 24 h as described previously (Bakre *et al.*, 2012) and expression levels of the 10 target genes chosen above were measured by qRT-PCR using gene-specific primers relative to 18S rRNA. miR-24 inhibitor transfection increased expression of PLK3, CSNK1G1, FGFR3 and KLF6 (Fig. 4c). Expression of these genes was significantly ($P < 0.05$) repressed in cells infected with Δ NS1 virus (data not shown). Sequence analysis showed that the 3' UTRs of CSNK1G1 harboured two miR-24-binding sites, whilst PLK3, FGFR3 and KLF6 each had a single miR-24-binding site (Fig. S2). These data suggest that NS1-mediated miR-24 suppression induced the expression

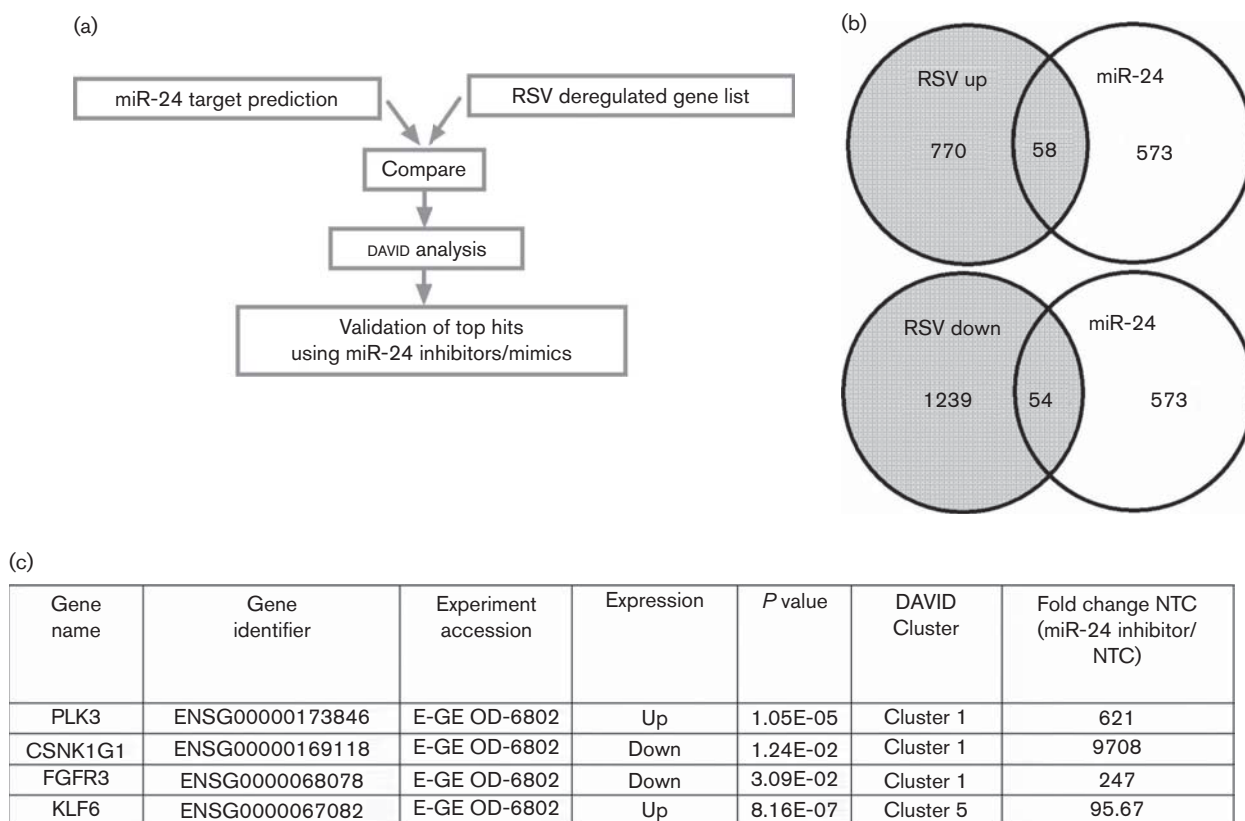


Fig. 4. miR-24 regulates multiple cellular pathways. (a) Work flow used to shortlist miR-24 target genes. Top target predictions were compared to public microarray data on RSV infection. (b, c) Subsets of genes (b) identified were validated using miR-24 inhibition experiments (c). A549 cells (2×10^5) were transfected with miR-24 inhibitor (25 nM) or mock or non-targeting control (NTC) for 24 h followed by analysis of gene expression using target-specific primers. Expression was calculated relative to 18S rRNA and non-targeting control from two or three independent experiments.

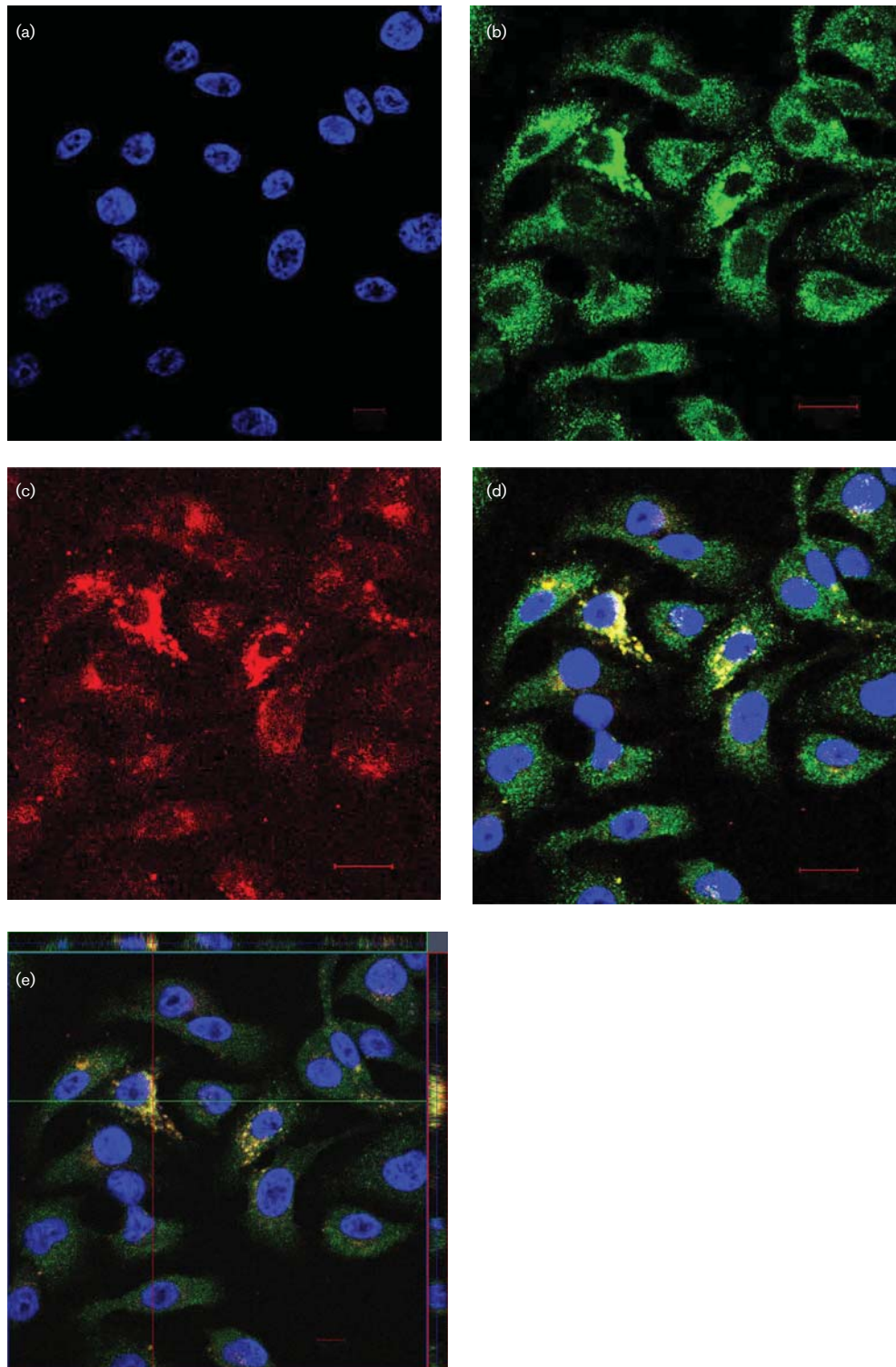


Fig. 5. RSV NS1 interacts with KLF6. A549 cells were transfected with 1.5 μg pEGFPC1-NS1 plasmid for 24 h in a 24-well plate and then transferred to chamber slides. Cells were allowed to adhere overnight, fixed, and stained for GFP expression using an anti-GFP antibody (green) and KLF6 (red). Nuclei were stained with DAPI ($1 \mu\text{g ml}^{-1}$). Confocal images were captured using a Zeiss LSM 710 inverted confocal microscope using an oil immersion lens at $\times 63$. Images are representative of three fields. (a) DAPI alone, (b) GFP alone, (c) KLF6 alone, (d) merged, and (e) orthogonal projection of the optical section showing co-localization of NS1 and KLF6. Bar, 10 μm .

of PLK3, CSNK1G1, FGFR3 and KLF6 via a miR-24 interaction with the cognate 3' UTR.

RSV NS1 co-localizes with KLF6

Given the finding that RSV NS1 induces KLF6 expression, which induces TGF- β to suppress miR-24 activity, the possibility that RSV NS1 interacted with KLF6 was examined in A549 cells. A549 cells expressing EGFP-NS1 stained for EGFP and KLF6 expression, and examined by confocal microscopy, showed that the fluorescence signals for NS1 overlapped with KLF6, suggesting that these proteins co-localize (Fig. 5d). To determine if this was the case, orthogonal optical sections were analysed in the *xy*, *xz* and *yz* planes to determine the localization of the fluorescence signals for NS1 and KLF6. The fluorescence signals for NS1 and KLF6 localized to the same positions, suggesting that these proteins co-localize and potentially interact (Fig. 5e). To validate if KLF6 and NS1 interact, co-immunoprecipitation assays were performed using an anti-GFP antibody to pull down KLF6 in EGFP-NS1-transfected A549 cells followed by a Western blot for KLF6. Co-immunoprecipitation failed to pull down detectable KLF6 by GFP pull-downs. This could have been due to either a genuine lack of direct KLF6 and NS1 interaction, or KLF6 and NS1 may have interacted transiently or indirectly through an accessory protein. Analysis of the KLF6 interactome using STRING database (Jensen *et al.*, 2009) identified NOP56 ribonucleoprotein (NOP56), cadherin 1 (CDH1) and histone deacetylase 3 (HDAC3), three proteins that have been identified previously as NS1-interacting partners (Wu *et al.*, 2012) (Fig. S3). These data suggested that NS1 may modulate the expression of KLF6 via interacting with these proteins.

DISCUSSION

Understanding how viral proteins modify host immune responses for viral survival and persistence is crucial to the development of effective countermeasures. Previous reports suggested that RSV non-structural proteins could modulate the expression of miRNAs during infection (Bakre *et al.*, 2012; Thornburg *et al.*, 2012). Here, it is demonstrated that a lack of NS1 during RSV infection significantly ($P \leq 0.05$) induced miR-24 expression in type II lung epithelial cells (Fig. 1a) and that overexpression of NS1 substantially suppressed miR-24 expression (Fig. 1b). Furthermore, it is shown that NS1 induces expression of KLF6, a positive regulator of TGF- β , and silencing KLF6 represses TGF- β production and viral replication, but induces miR-24 expression. Confocal microscopy identified co-localization of NS1 and KLF6 proteins, suggesting potential interaction. Although co-immunoprecipitation experiments did not adequately demonstrate NS1-KLF6 interaction, analysis of KLF6-interacting proteins identified three proteins (CDH1, HDAC3 and NOP56) that were reported previously to interact with NS1. These data support our proposed model where RSV NS1 suppresses miR-24 via

a KLF6/TGF- β -mediated pathway to affect multiple cellular pathways. It was shown previously that inhibiting miR-24 reduces RSV replication (Bakre *et al.*, 2012), and this may be a mechanism to ensure viral persistence and delay clearance. Two different genomic loci (19p13 as a miR-23b-27b-24 and 9q22 as part of miR-23a-27a-24-2) encode miR-24, but express a single mature miR-24 (Chhabra *et al.*, 2010). A549 cells lack the chromosome 19 locus (Xie *et al.*, 2013) and hence express miR-24 from the chromosome 9 locus only. Irrespective of origin, miR-24-1 and miR-24-2 transcripts have the same mature sequence, and thus the same target gene repertoires.

KLF6 belongs to the KLF family and encodes three isoforms ranging from 237 to 283 aa in length. KLF6 is expressed in the lung, and has been shown to be induced during infection by RSV and hepatitis C virus (Papic *et al.*, 2012), and by bacterial pathogens (Kidane *et al.*, 2013). In macrophages, KLF6 is induced by LPS and IFN- γ , and regulates macrophage polarization by suppressing peroxisome proliferator-activated receptor- γ (Date *et al.*, 2014). KLF6 also acts as a co-activator for NF κ B-regulated expression of p65-dependent pathways (Zhang *et al.*, 2014). In this study, silencing KLF6 significantly induced miR-24 expression, suggesting that KLF6 and miR-24 can interact in a bidirectional manner. RSV NS1 can modulate this interaction, as confocal microscopy indicated co-localization of KLF6 with RSV NS1 in cells transfected with plasmids expressing EGFP-tagged NS1. Furthermore, deletion of NS1 (rA2 Δ NS1EGFP) resulted in reduced KLF6 expression relative to WT RSV infection at 6 and 24 h p.i. (Fig. 2c).

In A549 cells infected with RSV, KLF6 regulates the induction of both TGF- β (Mgbemena *et al.*, 2011) and inducible nitric oxide synthase (Mgbemena *et al.*, 2013), inhibiting the cells in G₀/G₁ phase and preventing apoptosis during RSV infection (Mgbemena *et al.*, 2012). miR-24 inhibition is also known to block the cell cycle in A549 cells (Cheng *et al.*, 2005). Based on this evidence, it was hypothesized that RSV NS1 induces cell cycle arrest by promoting KLF6 transcription of TGF- β and inhibiting miR-24 expression. Indeed, the overexpression of NS1 induced elevated TGF- β expression. Treatment of NS1-expressing cells with either LPS or IFN- λ further elevated TGF- β expression. IFN- λ and also IFN- β treatment induced the expression of KLF6, which would also lead to elevated TGF- β . Treatment with IFN- λ causes cell cycle arrest in A549 (Li *et al.*, 2012) and other cell lines (Li *et al.*, 2008, 2010), and may do so via elevated TGF- β . The release of IFN- λ during infection may also induce TGF- β expression and cell cycle arrest in neighbouring uninfected cells during RSV infection. In this study, the link between TGF- β , KLF6 and miR-24 was demonstrated in A549 cells by the reduction in both TGF- β expression and RSV replication, and by the significant induction ($P < 0.05$) of miR-24 expression when KLF6 was silenced. Furthermore, treatment of A549 cells with TGF- β suppressed miR-24 and induced KLF6, indicating a feed-forward loop to induce

further TGF- β expression. TGF- β has been shown previously to regulate miR-24 expression (Sun *et al.*, 2008).

These data demonstrate a mechanism by which RSV induces cell cycle arrest in infected cells via TGF- β expression, which is promoted by NS1-mediated inhibition of miR-24 and elevation of KLF6 expression. Inhibition of miR-24 has also been shown to deregulate the cell cycle in A549 cells (Cao *et al.*, 2012; Cheng *et al.*, 2005) by inhibiting the anti-apoptotic factor XIAP (X-linked inhibitor of apoptosis), a protein induced during RSV persistence (Nakamura-López *et al.*, 2011). However, cellular processes other than cell cycle arrest are also affected by inhibition of miR-24. Recently, miR-24 was shown to modulate the replication of highly pathogenic influenza H5N1. Inhibition of miR-24 induced furin activity and increased viral replication for H5N1, but not H1N1 viruses (Loveday *et al.*, 2015). As furin is also involved in cleavage of RSV F (Basak *et al.*, 2001; Bolt *et al.*, 2000; Sugrue *et al.*, 2001), it is possible that downregulation of miR-24 may induce furin activity for efficient conversion of the RSV F0 to F1/F2 forms required for adhesion and formation of RSV filaments (Krzyzaniak *et al.*, 2013). miR-24 also regulates the processing of the pre-TGF- β 1 isoform via furin (Luna *et al.*, 2011), such that reduced miR-24 expression would lead to elevated furin-mediated TGF- β 1 processing. TGF- β 1 induces miR-23 but not miR-24 expression in A549 cells and suppresses miR-24 in myocytes, both in a Smad3-dependent manner (Cao *et al.*, 2012).

In summary, this study identifies for the first time, to the best of our knowledge, a mechanism by which RSV NS1 suppresses the expression of miR-24 and induces KLF6 expression to promote TGF- β -mediated cell cycle arrest and RSV replication. miR-24 may prove to be a viable antiviral target, as the induction of miR-24 may overcome RSV-induced cell cycle arrest and inhibition of apoptosis, thus improving viral clearance from infected cells.

METHODS

Cell culture, virus propagation and recombinant TGF- β treatment. A549 cells (ATCC CCL-185) were cultured in Dulbecco's modified Eagle's medium (DMEM) (HyClone) containing 5% heat-inactivated FBS (Hyclone) (Bakre *et al.*, 2012; Oshansky *et al.*, 2009a). Mycoplasma-free stocks of RSV strain A2 and those lacking the NS1 or NS2 genes (ra2 Δ NS1EGFP or ra2 Δ NS2EGFP, respectively) (Webster Marketon *et al.*, 2014) were expanded in Vero E6 cells (ATCC CCL-81) and maintained in DMEM (Hyclone) supplemented with 5% heat-inactivated FBS (Hyclone) as described previously (Bakre *et al.*, 2012; Oshansky *et al.*, 2009a). Recombinant TGF- β (Peptrotech) was reconstituted in 10 mM citric acid buffer as per the manufacturer's instructions. Plasmids pEGFPC1-NS1 and pEGFPC1-NS2 were grown in *Escherichia coli* TOP10 and isolated using standard protocols.

miRNAs, cell transfection and cell cytotoxicity. All transfection procedures were performed in triplicate for at least three independent experiments. A549 cells (2×10^4 cells per well) were plated in 96-well Costar flat-bottom tissue culture plates (Corning) for 12 h at 37 °C and subsequently transfected for 18 h with 25 nM miRNA-specific

inhibitor or mimic, or non-targeting miRNA mimics or inhibitor to *Caenorhabditis elegans* miR-67 (inhibitor negative control; Dharmacon) using DharmaFECT 1 (ThermoFisher) as per the manufacturer's instructions. Cell cultures were evaluated post-transfection for cell cytotoxicity using Alamar blue dye (Serotec). Alamar blue fluorescence reduction was measured at the end of 1.5 h incubation using a Tecan Safire X2 at excitation 530 nm/emission 590 nm as per the manufacturer's recommendations.

RNA isolation and qRT-PCR. Total RNA was isolated using a RNAzol RT kit (MRC Gene) as per the manufacturer's protocol. Briefly, following transfection, infection or treatment, cells were harvested in RNAzol RT reagent and lysed by repeated pipetting. DNA, proteins and carbohydrates were precipitated, and purified RNA quantified and treated with RQ1 RNase-free DNase I (Promega). Then, 100 ng DNase-treated RNA was used for cDNA synthesis using a miRNA cDNA synthesis kit (Agilent) as per the manufacturer's protocol, diluted as required, and used as template for qRT-PCRs with gene-specific primers (Table S1) and SYBR Green-based Maxima ThermoScript qPCR master mix (ThermoFisher) in an Agilent Mx3000P or Mx3005P instrument with 5 μ M final concentration for both forward and reverse primers. Oligonucleotides were obtained desalted from IDT Biosciences and reconstituted to 10 μ M working concentrations. Cycling conditions were: initial denaturation 95 °C/10 min, 40 cycles of 95 °C/15 s, 60 °C/30 s and 72 °C/30 s, followed by denaturation curve analysis. Annealing temperatures were pre-optimized using gradient PCRs. Data shown are mean \pm SEM from three biological replicates with three replicates each.

Confocal microscopy. A549 cells were transfected with 100 ng pEGFPC1-NS1 plasmid using Lipofectamine 2000 (Invitrogen) as per the manufacturer's suggested protocol. Transfected cells were trypsinized, transferred to Lab-Tek II chamber slides (Nunc) and incubated overnight at 37 °C to allow for cell attachment. Cells were fixed with 4% formaldehyde (ThermoFisher) in PBS for 10 min and permeabilized using 0.1% NP-40 (Sigma) in PBS for 10 min. Fixed and permeabilized cells were blocked using 5% BSA (Sigma) and subsequently stained for GFP using mouse anti-GFP antibody (Abcam) coupled to Alexa Fluor 488 (Life Technologies). KLF6 was detected using rabbit anti-KLF6 polyclonal antibody (Abcam) coupled to Alexa Fluor 546 (Life Technologies). Nuclei were stained using DAPI (Life Technologies) (1 μ g ml⁻¹) for 10 min. Slides were mounted in Prolong Gold Anti-Fade (Life Technologies) and stored in the dark at room temperature for drying, followed by storage at 4 °C. Confocal laser scanning microscopy was performed using a Zeiss LSM 710 instrument. Z-stacks, and images were captured and analysed using Zen Black 2012 software (Zeiss). Scale bar=10 μ m.

Statistics. Statistical analysis was performed using GraphPad Prism version 5.0 using one/two-way ANOVA. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P < 0.0001$.

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