3 Screening for host factors involved in the restriction of influenza virus, using *in vitro* assays and knockout mouse models.

3.1 Introduction

The use of knockdown technology has proven to be an invaluable tool in investigating the actions of innumerable host proteins in development, homeostasis and immunity (Mohr *et al.* 2010). It also provides a crucial first step in identifying putative targets whose expression can be ablated in a model organism, which can be more insightful and revealing than studying cell lines in isolation.

RNA interference (RNAi) is a eukaryotic cell technique based on a system that exists in mammalian cells to regulate gene expression and RNAi has been utilised to reduce the expression of specific genes in cells and organisms (Kim and Rossi 2008). The premise of the technique is that specifically designed short interfering RNA molecules (siRNAs) of about 21-22bp in length bind to a specific site on mRNA, which ultimately stimulates the cell to enzymatically degrade the mRNA; thus preventing translation (Fire *et al.* 1998).

The advantages of *in vitro* RNAi technologies are that it is very quick and economical to gather large amounts of information about the actions of potentially every gene in a targeted genome under a particular physiological condition, such as differentiation, tumorigenesis, or pathogen infection (Kamath *et al.* 2003; Boutros *et al.* 2004; Westbrook *et al.* 2005; Brass *et al.* 2009). The use of these high-throughput screening (HTS) techniques at the organismal level in "simple" model organisms such as *Caenorhabditis elegans* and *Drosophila melanogaster*, removes the need for gene trapping and mutagenesis, which are exploited in other more "complex" model organisms. Indeed, it is now possible to target genes in a tissue specific manner in *D. melanogaster* (Dietzl *et al.* 2007); thus further expanding the utility of RNAi technology in understanding gene function. Furthermore, this again demonstrates the relative simplicity of these model organisms, compared with mice, which would require the time-consuming generation of lines with tissue-specific Cre-drivers that have to then be crossed with an appropriate transgenic mouse with *LoxP* sites (Gu *et al.* 1994).

Recently, a number of studies have been published that utilise RNAi technology to analyse the host-virus interactions that occur at the cellular level, in both insect (Hao *et al.* 2008a) and mammalian systems (Brass *et al.* 2009; Shapira *et al.* 2009; Karlas *et al.* 2010; Konig *et al.* 2010). These studies have identified several candidate virus replication dependence factors (VRDFs): host proteins that are exploited by the virus during replication, and viral restriction factors (VRFs): host proteins that prevent viral replication (Wash *et al.* 2012). Although the methodologies employed by such studies vary by cell type, screening technique and viral subtype, there is a degree of overlap, with some host factors being identified in several of the studies. Such cross-study confirmation would suggest that these host proteins should be more thoroughly investigated at the cellular and organismal level.

Whilst it may be useful to analyse the roles of these genes in *C. elegans* and *D. melanogaster*, such findings may not be directly applicable to humans, although some cross-over between insect and human screens is evident (Figure 1.10). In order to explore the relevance of these genes to human health, it is appropriate, in addition to RNAi, to employ knockout mouse models, as they are amenable to genetic manipulation and serve as an appropriate organism for influenza virus challenges (discussed in section 1.5).

Although there was a degree of consensus amongst the influenza RNAi studies, it is accepted that false positive results can be reported through screen noise, experimental duration and analysis techniques (Mohr *et al.* 2010). Therefore, the aim of this set of studies was to first validate a number of the key targets identified by performing my own small scale RNAi studies, and simultaneously screen some of the currently available knockout mouse lines that have been generated at the WTSI, as part of the Mouse Genetics Programme. These particular lines have been selected as the literature, either through HTS RNAi screening or individual experiments, would suggest a phenotypic effect upon exposure to influenza virus.

3.1.2 Targets for validation of antiviral function

3.1.2.1 ARCN1

Archain 1 (*ARCN1* / *COPD*) is the δ portion of seven-subunit coat protein I (COPI) coatomer complex (Kirchhausen 2000). The primary function of the COPI complex concerns intracellular

trafficking of vesicles between the ER and Golgi: a cell function that is utilised by influenza virus during its replication cycle. The *ARCN1* gene was identified as being involved in the influenza virus replication cycle in three of the currently available HTS studies (Brass *et al.* 2009; Karlas *et al.* 2010; Konig *et al.* 2010). These findings would suggest that ARCN1 is a VRDF and that its knockdown restricts influenza virus infection.

3.1.2.2 CALCOCO2

Calcium-binding and coiled-coil domain-containing 2 (*CALCOCO2* / *NDP52*) is a dimeric, multimeric, cytoplasmic and nucleus-associated protein (Sternsdorf *et al.* 1997), which is thought to primarily be either a) a negative regulator of secretion (Morriswood *et al.* 2007), or b) acting as a receptor for ubiquitin-tagged proteins within endosomes. The detection of ubiquitin by CALCOCO2 is thought to result in autophagy by the cell (Thurston *et al.* 2009). It is capable of restricting bacterial infection in cells through the recruitment of various other host proteins to commence autophagy (Thurston *et al.* 2009; Thurston *et al.* 2012; Watson *et al.* 2012). The *CALCOCO2* gene was identified as being involved in the influenza replication cycle by two of the currently published HTS studies (Brass *et al.* 2009; Shapira *et al.* 2009), where it is suggested that CALCOCO2 is a VRDF, wherein its knockdown restricts influenza virus infection.

3.1.2.3 COPG

Coatomer subunit gamma (COPG) forms the γ subunit of the COPI complex, which ARCN1 is also associated with (COPG and ARCN1 functions are discussed in sub-section 3.1.2.1). Similarly, COPG was identified in three of the currently published HTS studies (Brass *et al.* 2009; Karlas *et al.* 2010; Konig *et al.* 2010) and is thought to act as a VRDF.

3.1.2.4 IDO1

Indoleamine 2,3-dioxygenase 1 (*IDO1* / *INDO*) is an enzyme involved in tryptophan catabolism and is thought to have an important immunomodulatory role in the host through its influence on T-cell apoptosis (Munn *et al.* 1999) and may also form a crucial component of the innate immune system of the cell, owing to its interactions with STAT1, IFNγ and TNFα (Chon *et al.* 1996; Adams *et al.* 2004). IDO1 has thus far been implicated in the control of multiple pathogenic viruses, including vaccinia virus, West Nile virus, murine leukaemia virus (MLV)

and hepatitis B virus (Terajima and Leporati 2005; Hoshi *et al.* 2010; Mao *et al.* 2011; Munoz-Erazo *et al.* 2012), as well as being linked with the development of a number of cancers (Uyttenhove *et al.* 2003; Liu *et al.* 2010b; Bonanno *et al.* 2012). Although IDO1 was not revealed as a candidate by the HTS studies, it has been included in the study owing to its known role in the life-cycles of a broad range of viruses, much like IFITM3 (Brass *et al.* 2009), and evidence indicating it may have a role in influenza virus infection through its role in inflammation (van der Sluijs *et al.* 2006).

3.1.2.5 SMS

Spermine synthase (*SMS* / *SPS*) is an enzyme that converts spermidine and S-adenosylmethionine into spermine. Spermine, a polyamine, has multiple roles in cells; influencing cell growth, differentiation and cell death (Wallace *et al.* 2003). As with *IDO1*, *SMS* was not a hit in the HTS studies, but has been included owing to a potential role of spermine in the inactivation of multiple viruses, including influenza, West Nile and vaccinia viruses (Bachrach 2007).

3.1.2.6 TM9SF4

Transmembrane 9 superfamily protein member 4 (*TM9SF4*) is a nonaspanin molecule, associated with the endosomal membrane. Although the exact function of the TM9SF family is unknown, it is becoming apparent that these proteins show strong evolutionary conservation amongst animal species (Pruvot *et al.* 2010). TM9SF4 plays a role in cell adhesion, as well as in macrophage engulfment of pathogens, with knockout *D. melanogaster* showing a depleted ability to engulf Gram-negative bacteria (Bergeret *et al.* 2008). Interestingly, TM9SF4 has also been linked with tumour cannibalism in humans, wherein it is seen to directly interact with RAB5A in the endosomal membrane; its silencing was shown to directly influence the acidification of the endosomal compartment (Lozupone *et al.* 2009). Although TM9SF4 has not been shown to be a target in the influenza HTS studies, it is a VRDF in West Nile virus HTS studies (Krishnan *et al.* 2008). Furthermore, its role in endosomal acidification, which is exploited by influenza virions, and its putative role in phagocytosis potentially suggest it may have a role in influenza virus replication.

3.2 Results

3.2.1 The impact of gene knockdown on susceptibility to influenza virus infection in human cell lines

To investigate the effects of the loss of translation directed by mRNA from the genes listed in sub-section 3.1.2 on influenza virus infection, RNAi studies were conducted using two human cell lines: U2-OS and A549 cells. Although A549 cells are the more relevant cell line, owing to being derived from lung epithelia, they also lack the expression of IFITM3 (data not shown), a crucial antiviral restriction factor. Therefore, U2-OS cells were also used to investigate the effects of the gene knockdown. Cells were transfected in duplex with siRNAs (Table 2.6) and subsequently infected with WSN/33 influenza virus.

As shown in Figure 3.1, the siRNAs had varying effects on influenza infectivity. The screening revealed that the greatest effects were elicited from the silencing of *ARCN1* (A549, $p < 0.001$; U2-OS, $p < 0.001$) and *COPG* (A549, $p < 0.001$; U2-OS, $p = 0.003$) in both cell lines, which significantly reduced influenza virus replication over the 18 hours of infection. This was similar to the effect observed when cells were treated with siRNAs specific to the influenza virus' *NP* (A549, $p < 0.001$; U2-OS, $p < 0.001$), which are intended to impede influenza replication and act as a positive control for reduction of virus replication. Interestingly, a mild, but significant reduction in infectivity in U2-OS cells was also observed when *SMS* was silenced ($p = 0.003$), but this effect could not be seen in A549 cells.

Conversely, the reduction in expression of IDO1 resulted in a small, but significant increase in viral replication in both cell lines (A549, $p = 0.012$; U2-OS, $p = 0.011$). However, no statistically significant alterations in viral infectivity was observed with siRNAs designed against *CALCOCO2* or *TM9SF4*.

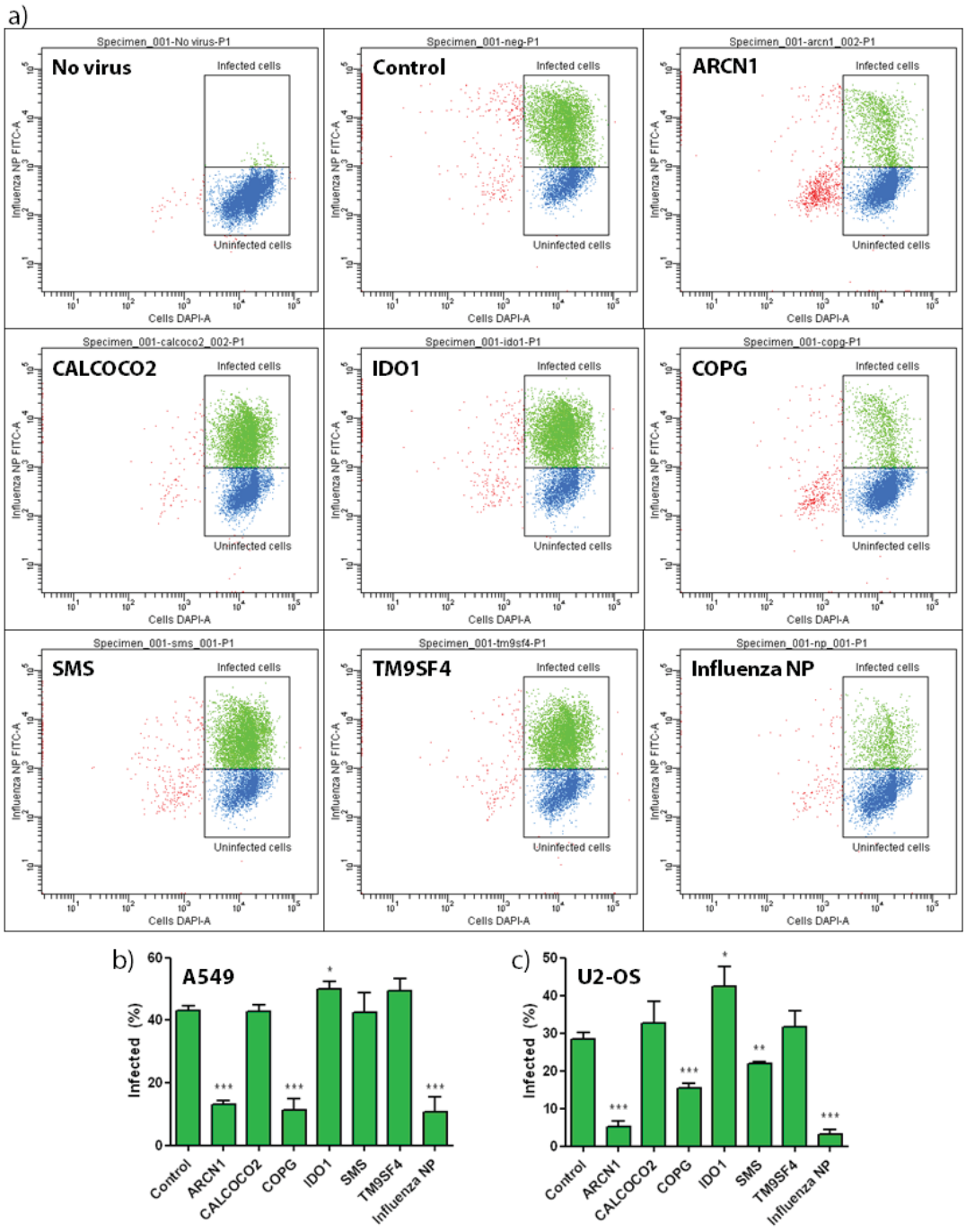

Figure 3.1: The impact of gene knockdown on influenza infection in A549 and U2-OS cell lines. Human cell lines were transfected in duplex with the indicated siRNAs for 48 hours, after which they were infected with WSN/33 influenza virus (MOI = 0.1 PFU/cell) for 18 hours before being assayed for infectivity by flow cytometry. Representative A549 flow cytometry profiles (**a**) for each of the siRNAs tested in the study are shown. Cells were double stained with Hoescht (cell nuclei) and FITC (influenza NP). Mean percentage of A549 (**b**) and U2-OS (**c**) cells staining positive for influenza virus are also shown. Results show means from >3 biological repeats ± S.D. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

Gene expression and related knockdowns were confirmed by RT-qPCR analysis 48 hours after transfection, which showed that on average the targeted genes were reduced to 14% the expression of control cells receiving scrambled siRNAs (Table 3.1).

Table 3.1: Percentage expression of targeted genes in A549 cells following siRNA knockdown.

	siRNA target gene					
	Arcn1	Calcoco2	Copg	Ido1	Tm9sf4	Sms
Percentage of "normal" expression:	4.2	14.8	24.3	21.2	5.5	13.9

All percentages are relative to cells transfected with control scrambled siRNAs.

3.2.2 The impact of gene knockout on susceptibility to influenza virus infection in mouse knockout lines.

Several of the candidate genes listed in section 3.2.1 have been knocked out in C57BL/6 mice as part of the WTSI's Mouse Genome Project. To test the effects of the loss of genes *in vivo* during influenza virus infection, mice were intra-nasally challenged with 10^4 PFU of A/X-31 influenza: a sub-lethal dose, and monitored for 10 days for signs and symptoms of disease. Some groups of mice were also re-challenged 3 weeks later to test for any defects in their humoral and cellular adaptive immune response.

The mouse lines available for infection over the course of these studies were those with mutations of *Arcn1*, *Calcoco2*, *Copg*, *Ido1*, *Sms* and *Tm9sf4*. However, difficulties in breeding meant that not all lines could be challenged with influenza virus: *Sms* knockout mice were homozygotic lethal in females, which led to an insufficiently large colony; *Ido1* knockout mice bred poorly, which also led to an insufficiently large colony; and *Arcn1* knockout mice were shown to be incorrectly targeted at the gene level, with reportedly $Arcn1^{-/-}$ mice possessing an intact *Arcn1* allele.

Homozygous mice ($n < 5$ per genotype) with ablations of *Calcoco2* and *Tm9sf4* were challenged with influenza, but showed no statistical difference in weight loss profiles (Figure 3.2a,b), indicating there was no effect of these mutations. $Copg^{-/-}$ mice were homozygotic lethal, so $Copg^{+/-}$ mice were challenged to test for heterozygotic effects. Similarly, no statistically

significant phenotype was detectable in these mice (Figure 3.2c). All mice also showed no loss of weight loss of morbidity when re-challenged with A/X-31 influenza.

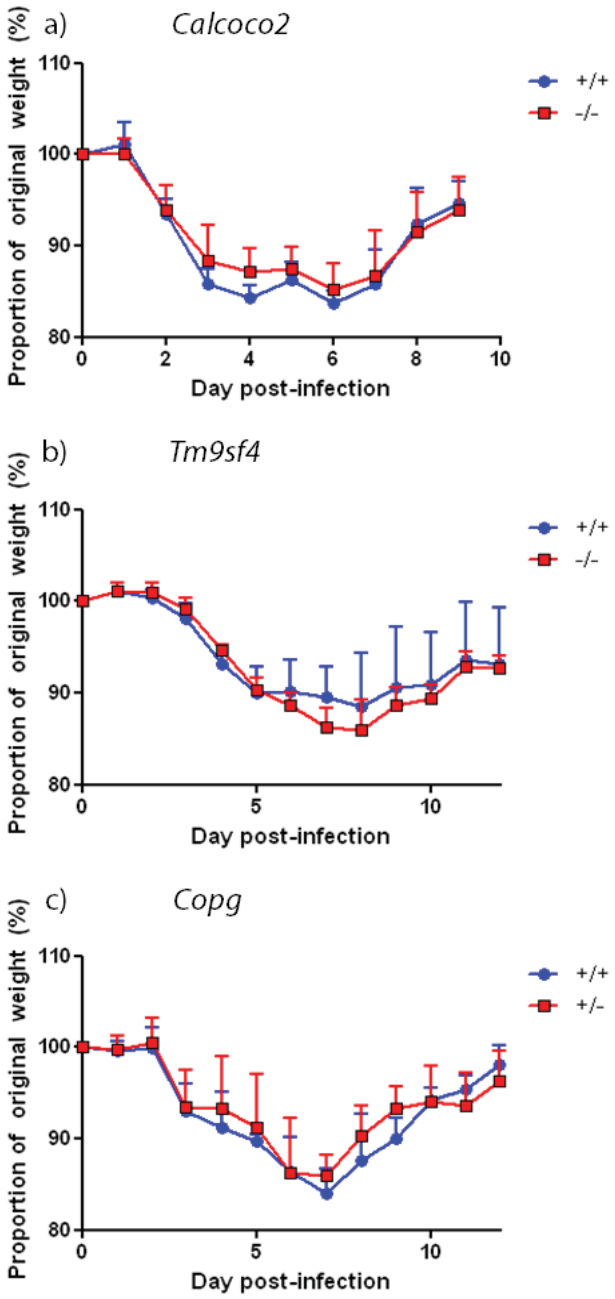

Figure 3.2: Weight loss profiles of knockout mice screened for susceptibility to influenza virus infection. Mice with specific deletions in their a) *Calcoco2*, b) *Tm9sf4* and c) *Copg* alleles were infected intra-nasally with 10^4 PFU of A/X-31 influenza virus, weighed daily and monitored for signs of morbidity. Results show means from n > 5 mice per genotype, ± S.D.

3.3 Discussion

In this study, I explored the effects of a small set of gene knockdowns in both human cell lines and mice. The body of literature generated through HTS studies has suggested that numerous host proteins inhibit or promote viral replication *in vitro*. The aim of this pilot study was to validate these screens in order to ascertain whether they may yield a phenotype in knockout mouse lines.

The strongest phenotypic effects seen in the study were from ARCN1 and COPG, which form two portions of the COPI complex, which is involved in retrograde transport of vesicles between the ER and Golgi apparatus. Highly significant p-values ($p < 0.001$) were attained for siRNA-mediated knockdowns in both A549 and U2-OS cells. These findings ratify those reported in previous HTS studies (Brass *et al.* 2009; Karlas *et al.* 2010; Konig *et al.* 2010), where they produce a significant reduction in influenza virus infectivity. Since the current study was conducted, Sun and colleagues (2012) investigated the specific role of the COPI complex in relation to influenza infectivity. They too showed that siRNA-mediated depletion of ARCN1 resulted in a significant reduction in influenza virus replication; concluding that the COPI complex was involved in indirectly influencing vesicle trafficking in the late endosomal stages. Furthermore, they also inhibited the COPI complex via pharmaceutical intervention, which uncovered a role in potentially assisting in viral membrane formation prior to budding. Although my findings are consistent with those published in the literature, I also observed significant cell death as a result of the siRNAs directed against ARCN1 and COPG. Such cytotoxicity was not observed in any other *in vitro* knockdowns, which would suggest that these genes are intrinsic to cellular viability. Interestingly, this was not reported in previous studies looking at depletion of the COPI complex by siRNA (Brass *et al.* 2009; Karlas *et al.* 2010; Konig *et al.* 2010; Sun *et al.* 2012), but was observed in all independent repeats by both myself and colleagues at the WTSI. However, it is possible that such differences are due to internal practices and experimental setup, and may not be as a result of the knockdown.

However, the attempts to generate mice with a gene ablation in the COPI complex would suggest that these genes are essential for survival. *Copg*$^{-/-}$ mice proved to be homozygotic lethal; therefore impeding the study as I could only monitor heterozygous mice, which yielded no

significant obvious deviations from the normal progression of influenza virus infection. In order to investigate the effects of complete ablation of *Copg* under influenza challenge, I sought to acquire and cross the *LoxP*-containing *Copg*$^{+/-}$ mice with those possessing a CCSP-driven Cre allele (Bertin *et al.* 2005). CCSP (Clara cell secretory protein) is specifically expressed in the respiratory tissues, and as such would result in the excision of the remaining *LoxP*-flanked *Copg* allele. However, we entered difficulties in acquiring the Materials Transfer Agreement (MTA); thus making the transgenic generation infeasible. The WTSI will now attempt to generate this mouse on-site for future lung-specific gene deletions.

The impact of the loss of *IDO1* in my screen was also notable. The knockdown of *IDO1 in vitro* resulted in a small, but significant ($p<0.05$) increase in viral infection in both A549 and U2-OS cells, which would suggest IDO1 is an antiviral molecule. Previously, HTS results have shown that knockdown of IDO1 results in increased infectivity with West Nile virus and chikungunya virus *in vitro* (Schoggins *et al.* 2011), but this presents the first evidence that IDO1 may impact upon influenza virus. Although the *Ido1*$^{-/-}$ mouse line is viable and available at the WTSI, sufficient numbers of mice were unavailable to perform a robust series of experiments. However, the current RNAi evidence indicates that this line should be pursued as a priority, as it may yield a positive phenotype. This is especially true when considering the potential role of IDO1 in moderating T-cell responses (Munn *et al.* 1999; Larrea *et al.* 2007) and in the stabilisation of mRNA coding for pro-inflammatory cytokines such as IL-6 (van Wissen *et al.* 2002). It is anticipated that the *Ido1*$^{-/-}$ mouse will show increased pathological damage and heightened viral replication when challenged, but confirmatory data is awaited.

Of the knockout animals challenged in this study, *Calcoco2*$^{-/-}$ mice represent the only viable homozygotes to be represented in multiple HTS studies for influenza infectivity. However, I was unable to replicate the results found in previous studies, with the knockdown of *CALCOCO2* in cell lines resulting in non-significant differences compared to control cells. Similarly, the mice challenged with influenza virus showed a marginal trend towards protection from the virus, but these differences were also non-significant. Interestingly, sequence alignment of the human (AAH15893) and mouse (NP_001257949) proteins using ClustalW2 (Larkin *et al.* 2007) revealed only 39% similarity at the amino acid level. It may therefore not be surprising to note

that the published effects of CALCOCO2 knockdown in cell lines are not replicated in mice, owing to divergent evolution.

This study highlights several key issues that must be considered with both *in vitro* assays and the use of knockout mouse lines. Perhaps the most evident problem is that *Copg*$^{-/-}$ mice have a lethal phenotype. HTS studies, and this RNAi work, would suggest that the ablation or knockdown of the COPI complex would result in reduced influenza virus infection. One could therefore surmise that this complex may be a viable target for antiviral interventions. However, the observation that these cells exhibited increased rates of cell death and that *Copg*$^{-/-}$ mice were non-viable (one would presume the same to be true for the *Arcn1*$^{-/-}$ mice should they have been generated appropriately), would suggest this may not be the case. Whilst RNAi screens are useful for understanding cellular dynamics and viral replication, as well as focusing attention onto certain genes, they may also focus research onto areas that have no translational potential to human therapies.

The second, more general problem, stems from the use of knockout mouse lines to mimic the situation seen in humans. The discrepancies that were observed between the effects of the loss of *Calcoco2* in mice and the knockdown of *CALCOCO2* in HTS studies with human cells may be a result of divergence between the orthologs, which may have resulted in altered functionality. Previous comparisons of the genetic and amino acid sequences of human and mouse orthologs have revealed approximately 80-85% sequence similarity (Batzoglou *et al.* 2000; Chinwalla *et al.* 2002). Therefore, the poor degree of sequence homology here (39%) would indicate that the *Calcoco2*$^{-/-}$ mouse may not be an accurate model for the study of the effect of silencing the human gene *in vivo*. Such extensive sequence divergence should therefore be considered when comparing across species barriers.

In conclusion, HTS studies are an important tool in rapidly identifying genes that may be involved in cellular processes, and in particular those involved in influenza infection cycles. Whilst the current study encountered some discrepancies with the published literature, they illustrate how it is possible to translate *in vitro* into *in vivo* studies through the use of knockout animal models, such as mice. Ultimately, the goal of such progression would be to take a further

step and utilise the information ascertained from cell lines and mice through to human studies. Such translational research could inform potentially important diagnostic and therapeutic tools.