

## **CHAPTER 7: Future Directions**

## ***In Situ* Hybridisation Screen for Developmentally Regulated Ubiquitin Modifying Enzymes**

The full potential of the ISH screen for developmentally regulated ubiquitin modifying enzymes described in Chapter 3 was not realised and the possibility remains that the expression of some ubiquitin pathway enzymes may be restricted during development due to participation in specific developmental events. Should this screen be revisited in the future, there are a number of ways in which it could be improved. The greatest improvements can be made in the identification of ubiquitin pathway enzymes, preselection of those targets that are most likely to be expressed at ISH detectable levels and standardising the procedure for probe selection and synthesis.

In the past five years considerable successes have been made in sequencing complete genomes, including the mouse genome. Murine sequence data, which is being progressively annotated, is publicly available through institutions such as NCBI and ENSEMBL and may be queried using databases such as Entrez Gene and Unigene (Maglott et al., 2005; Wheeler et al., 2005). The vast increase in knowledge of the mouse genome is of obvious use in identifying ubiquitin pathway enzymes, particularly those that are identifiable by characteristic motifs such as the USP class of deubiquitylating enzymes. For the study described in Chapter 3, cDNA was acquired for 13 distinct USPs. Searching the Entrez Gene database reveals that these cDNAs represent little over one quarter of the 49 genes that are confirmed or predicted members of this enzyme class and have been systematically assigned numbers from USP1 to USP54. Although not all ubiquitin modifying enzymes can be identified by sequence motifs, gene annotation allows for identification of genes that have been functionally shown to participate in the ubiquitin pathway. The Unigene database also contains expression profiles compiled from EST libraries, and this data could be used to pre-screen ubiquitin pathway enzymes for those that are most likely to be detected by ISH in the early post-implantation mouse embryo. Hence, increased knowledge of the genome allows for both the identification of a more comprehensive list of ubiquitin pathway enzymes and the refining of this list to target genes that are most appropriate for the method of investigation.

Screening the expression patterns of the target genes by ISH would also be improved by further standardisation of the process of cDNA acquisition and probe synthesis. Previously, cDNA was primarily acquired by request and consequently different cDNAs were received in different vectors. ISH probes were synthesised by *in vitro* transcription of DIG-labelled RNA probes from T7, T3 or SP6 Polymerase sites present on the vector backbone, with the transcription “stop site,” and length of the probe, being determined by prior linearisation of the vector. Due to the non-standardised nature of the cDNA templates, there was a significant variation in probe length for different genes, which could potentially result in variation of hybridisation efficiency and/or background hybridisation of the different probes. Furthermore, in some instances transcribed vector sequence can also lead to variation in the background hybridisation of a probe (Witkiewicz et al., 1993). To reduce this potential variation, probe sequences of a defined length (eg. 400bp) could be designed for each target gene, from available sequence data. Template cDNA for probe synthesis could subsequently be cloned from ES cell or embryonic cDNA libraries by PCR, with the introduction of appropriate sites for linearisation, and inserted into a vector with appropriate polymerase binding sites (eg pGEMTeasy, Promega). Designing two distinct probes for each target gene would add further rigour to the study and increase confidence in the genuine nature of observed expression patterns in the case of ubiquitous or absent staining.

The experimental protocols used for probe synthesis and *in situ* hybridisation compare favourably with published protocols (Abraham, 2001; Correia and Conlon, 2001), and do not require modification. However, trouble-shooting modifications may sometimes be appropriate. The most common cause of failure of the probe synthesis reaction is contamination of the DNA template with trace amounts of RNase. This problem can be eliminated by Proteinase K digestion of the DNA template, followed by phenol-chloroform extraction, prior to the synthesis reaction (Abraham, 2001). When conducting ISH, if there is doubt regarding whether a staining pattern is genuine or results from background hybridisation this question may be resolved by repeating the experiment with an RNase A treatment in between hybridising the RNA probe and detection of the probe (Correia and Conlon, 2001). Double-stranded RNA, such as appropriately bound probe will be resistant to RNase A digestion, while single-stranded RNA, such as background hybridised probe, will be digested. Hence background staining, but not genuine staining, will be reduced by RNase treatment.

In conclusion, for the purposes of this thesis, a screen for developmentally regulated ubiquitin modifying enzymes was not pursued following some ambiguous initial results. Greater knowledge of the mouse genome today would allow the identification of a larger, but more select, set of target genes. Applying a similarly designed, but more rigorous, ISH screen of these target genes during early post-implantation embryogenesis, would likely yield more significant results.

## **Analysis of the Role of the Deubiquitylating Enzyme FAM in Epithelia**

### ***FAM Gain of Function Analysis***

This thesis has described several approaches to the analysis of the role of FAM in epithelia through gain of function methodology. Although increased FAM protein levels have been demonstrated in transiently transfected HEK293T, little success has been achieved in generating stable FAM gain of function models. A stable FAM gain of function model was pursued as the epithelial cell line chosen for these studies, MDCKII, are transfected at low efficiency by liposomal DNA delivery reagents. Hence, the high proportion of unaltered cells in a transiently transfected population of MDCKII cells will obscure analysis of the effect of FAM gain of function on the whole population.

It was endeavoured to overexpress FAM in MDCKII cells using two unconditional overexpression systems and an inducible expression system. In the first unconditional expression system a single expression cassette drove the expression of FAM-IRES-neomycin resistance gene mRNA from a chicken  $\beta$ -actin promoter, such that expression of the selectable marker was dependent on expression of FAM. Following transfection with this vector, and geneticin selection, very few resistant colonies were generated, and those that were could not be demonstrated by Western blot analysis to have greater FAM protein levels than geneticin resistant control cell lines. In the second unconditional expression system, V5 tagged FAM and the blasticidin resistance gene were expressed from separate expression cassettes on the same vector driven by the elongation factor-1 $\alpha$  promoter and the EM7 promoter, respectively. Many blasticidin resistant MDCKII colonies formed following transfection with this vector and blasticidin selection. However, screening of

individual clones by Western blot analysis revealed detectable expression of exogenous FAM-V5 in less than 8% of these clones. To confidently assess total levels of FAM it was necessary to first raise sera against FAM that would reliably recognise both canine and murine FAM protein. This was achieved by immunising rabbits with the catalytic domain of FAM fused to GST (GST-FAMCAT). Affinity purification of anti-FAMCAT antibodies from the sera was found necessary to adequately reduce confounding background reactivity (Chapter 5). Application of this antibody to Western analysis of the FAM-V5 expressing clones demonstrated that total FAM levels in these clones were not significantly greater than in wild type cells or blasticidin resistant control cell lines. The inability to generate FAM overexpressing cell lines using either of these two unconditional expression systems suggests that there is strong selective pressure against unconditional increases in FAM levels. That the exogenous protein was rarely detected in FAM-V5 transfected cell lines, and was only ever seen to be expressed at low levels suggests that in most instances the FAM-V5 expression cassette may have been subject to gene silencing.

FAM gain of function analysis in MDCKII cells was further pursued using an inducible expression system. In an inducible system the FAM expression cassette can be selected for in the absence of “natural selection” against increased FAM protein levels. A Gateway® cloning compatible version of the Tet-on inducible system (T-rex; Invitrogen) was chosen for this purpose. This system is based on the establishment of a parental cell line which stably expresses a tetracycline responsive transcriptional repressor (TetR). The parental cell line is then stably transfected with an expression cassette for the gene of interest, FAM, from which transcription is repressed by TetR. The addition of tetracycline to the culture media of these cell lines will relieve the transcriptional repression by TetR, and therefore induce the expression of the gene of interest. Although the TetR MDCKII cell line has successfully been used in the analysis of other genes of interest (personal communication; Rino Donato, PhD candidate, Child Health Research Institute, Adelaide, SA), FAM expression was empirically found not to be inducible using this system. Cell lines were analysed for the induction of FAM expression by quantitative real time PCR and by Western blotting, and neither FAM mRNA nor protein was seen to be induced. This observation has not been further investigated, but could be explained if TetR repression of FAM expression is not 100% efficient during cell line selection. If this were the case then the tetracycline responsive FAM expression cassette may be silenced in a similar manner as was observed for the unconditional FAM expression cassettes. Regardless of the reason

it has been clearly empirically shown that MDCKII cells appear resistant to increased FAM levels, where the gain of function methodology is based on cationic liposome or calcium phosphate mediated delivery of a DNA vector into the cell followed by selection for stable chromosomal integration of the vector.

Given the ability to transiently overexpress FAM in the HEK293T cell line, greater success in generating a FAM gain of function MDCK model may be possible with a more efficient gene delivery system. The most common alternatives to liposomal reagents are viral gene delivery systems, such as modified retroviruses and adenoviruses. Both these systems have previously been successfully applied to MDCK cells (Kim et al., 2002; Pastan et al., 1988; Yeaman et al., 1996; Zambon et al., 2000). However, given that the FAM coding sequence alone is 7.5kb, design of a FAM expression cassette is likely to be constricted by the retroviral packaging size limitations (Federspiel and Hughes, 1997) and therefore an adenoviral system which can accommodate larger transgenes may be more suitable. Alternatively, methodologies other than gain of function in MDCKII cells may be used to pursue the role of FAM in epithelia, as discussed below.

### ***Live imaging of FAM***

Although, transfection efficiency of MDCK cells by non-viral means is low, it is sufficient to conduct single cell assays on fluorescently labelled proteins. Indications from the HEK293T cell line, from which attempts to isolate stable FAM overexpressing cell lines were unsuccessful, are that FAM can be overexpressed by transient transfection. Expression of a fluorescently tagged FAM (eg GFP-FAM) would allow FAM to be observed at the cellular level in real time and opens the possibility of applying recently developed real time microscopy techniques to the study of FAM (Lippincott-Schwartz and Patterson, 2003).

The first question to be addressed with GFP-FAM would be the confirmation of FAM's localisation in MDCKII cells. Published data shows FAM to be associated with sites of cell-cell adhesion in MDCKII cells (Taya et al., 1998) and to be predominantly associated with the Golgi and endosomal compartments in many other cell types (Murray et al, 2004 and unpublished observations). If this is confirmed, it would be interesting to compare FAM's association with the E-cadherin/ $\beta$ -catenin adhesion complex in MDCKII cells and

other epithelial cell types. There is circumstantial evidence linking FAM to the trafficking of the E-cadherin/ $\beta$ -catenin adhesion complex (Murray et al., 2004), but this interaction is poorly characterised. Colocalisation of FAM and E-cadherin or FAM and  $\beta$ -catenin could be monitored under a variety of conditions (eg stimulation of E-cadherin internalisation by HGF treatment) to further characterise when and where FAM associates with this complex. It is also unknown if FAM directly interacts with E-cadherin, or whether they are simply components within the same complex. FRET (Fluorescence Resonance Energy Transfer) could be applied to answer this question, and has advantages over biochemical techniques in that information on the intracellular location where direct interactions between the two proteins occur is gained.

However, to conduct these experiments fluorescently labelled FAM expression plasmids must first be constructed. Unfortunately the simplest strategy for achieving this, cloning FAM using Gateway technology into commercially available GFP fusion vectors has not yielded competent GFP-FAM expression constructs. These commercially available vectors encoded the cycle3 GFP variant and, as discussed in Chapter 6, cloning FAM into a fusion vector encoding the EGFP variant may yield a competent GFP-FAM expression construct. This may be achieved by either converting a conventional EGFP fusion vector into a Gateway plasmid or by using conventional cloning techniques. Applying conventional cloning techniques to the FAM cDNA is complicated by the many restriction enzyme sites present within the 7.5kb coding sequence. However, using Gateway cloning will introduce the attB1 recombination site between the GFP and FAM coding sequences, encoding 8aa which may, or may not, have structural effects on the protein linker region between GFP and FAM. There is also an alternative vital tag to GFP and GFP-like proteins in the Lumio™ system (Invitrogen). This system requires expression of the protein of interest as a fusion with a tetra-cysteine motif. Membrane permeable Lumio™ reagent, which fluoresces only when complexed with the tetra-cysteine motif, is then added to the expressing cells to allow visualisation of the protein of interest. As FAM can be successfully expressed as a fusion with the 23aa V5/His tag at its C-terminus, a similar fusion of a 6aa tetra-cysteine motif would not be expected to adversely affect the protein's stability. However, the Lumio™ fluorescence system is not as well characterised and not as versatile as GFP and its variants, and obtaining a functional GFP-FAM expression construct would be preferable.

## ***FAM Loss of Function Analysis***

In recent years it has become possible to complement gain of function experiments in cultured cells with gene knockdown studies using siRNA (Hannon and Rossi, 2004; Huppi et al., 2005; Meister and Tuschl, 2004), and this technique has been successfully applied by others to MDCK cells (Benais-Pont et al., 2003; Ortiz et al., 2004). Two FAM target sequences, conserved between human and mouse, have been successfully used in siRNA knockdown experiments of FAM in HeLA cells (Chen et al., 2003), but neither of these sequences are conserved in the canine FAM sequence. Hence the use of siRNA in the canine MDCKII cell line would require designing and testing new siRNA constructs. The canine FAM (USP9X) sequence recently became available, simplifying the process of siRNA design. Appropriate siRNA targets can be identified according to established rules (Elbashir et al., 2001) which can be implemented by freely accessible online software (available at: [www.ambion.com](http://www.ambion.com)). Subsequently, a number of designed targets are chosen for empirical testing. These may be constructed by *in vitro* transcription and the double-stranded RNA 21mers directly transfected into eukaryotic cells, or DNA expression vectors encoding the target sequence as a hairpin RNA (shRNA) may be cloned and transfected into eukaryotic cells. Obviously at this stage the same obstacles need to be overcome regarding the introduction of siRNA, or siRNA expression constructs, into MDCKII cells as with the introduction of FAM overexpression constructs.

Several approaches may be taken to the introduction of siRNA into MDCKII. Two such approaches that are likely to yield high transfection efficiencies are detailed below. Tailoring of a peptide based transfection reagent, MPG, for the efficient delivery of siRNA has been described (Simeoni et al., 2003). This reagent has recently been commercially developed (EXPRESS-si) by Genospectra (Fremont, CA, USA) who have achieved up to 90% siRNA transfection efficiency in difficult to transfect cell lines such as terminally differentiated 3T3L1 adipocytes at a range of cell densities (genospectra, 2005). Although this reagent is yet to be tested in MDCKII cells, its low toxicity in other cell lines and simple mechanism of action (direct diffusion through the plasma membrane) means that it is likely to be more effective at siRNA delivery in MDCKII cells than liposomal transfection reagents. The second approach, which has been successfully demonstrated for MDCK cells, is the introduction of shRNA using retrovirus (Schuck et al., 2004). Retroviral delivery into dividing cells is highly efficient and if expression of the shRNA is

coupled with a selectable marker, cell populations in which every cell contains at least one active copy of the retroviral genome can be obtained. However, shRNA knockdown is not indefinitely maintained in these cells and knockdown efficiency will slowly decay over a period of weeks (Schuck et al., 2004).

Should a knockdown of FAM levels be achieved in MDCKII cells through siRNA technology, these cells would be analysed in a number of ways, including application of the assays described in Chapter 6. The key questions to be addressed are whether alterations in FAM protein levels affect cell-cell adhesive properties and whether such alterations are due to changes in stability or localisation of the E-cadherin/ $\beta$ -catenin complex. The dynamics of the E-cadherin/ $\beta$ -catenin complex could be examined both during endocytosis and during formation of adherens junctions. E-cadherin has previously been shown to be endocytosed and recycled in confluent steady-state MDCK cells using a biotinylation assay (Le et al., 1999). This assay could be adapted to examine whether decreased FAM protein results in an increased rate of E-cadherin turnover either at steady-state or when E-cadherin internalisation is stimulated, such as by HGF treatment (Fujita et al., 2002). This scenario would be expected if FAM's role were to oppose the ubiquitylation and internalisation of E-cadherin. Ubiquitin has regulatory roles at many points in protein trafficking and so if E-cadherin is a substrate of FAM, trafficking of newly synthesised and recycling E-cadherin may also be altered by FAM knockdown. Recovery of E-cadherin and  $\beta$ -catenin to sites of cell-cell contact could be monitored by fluorescence microscopy following calcium switch.

### ***FAM C1566S Mutant***

FAM is a member of the USP class of deubiquitylating enzymes that are identifiable by conserved histidine and cysteine motifs (Amerik and Hochstrasser, 2004; Wood et al., 1997). It is generally accepted that the conserved cysteine residue is essential for deubiquitylating activity and mutation of this residue in a member of the USP class will yield a catalytically inactive mutant (Cohen et al., 2003a; Gilchrist and Baker, 2000; Li et al., 2002b). Directed mutagenesis has been used to generate such a FAM mutant, FAM C1566S, but as yet this mutant has not been further characterised, due to technical difficulties in introducing expression constructs into MDCKII cells and in generating a fluorescently tagged FAM. This, presumably, catalytically inactive mutant will be of

obvious use in determining which of FAM's functions are dependent on its deubiquitylating activity. Of particular interest, if technical difficulties in transfecting FAM overexpression constructs could be overcome, would be examining the ability of murine FAM constructs (wild type and C1566S mutant) to rescue a phenotype observed following FAM knockdown in MDCKII cells with a canine specific siRNA.

### ***Other Experimental Systems***

This thesis and discussion has focused on establishing an experimental model for examining the role of FAM in cell-cell adhesion regulation, in the MDCKII epithelial cell line. Although MDCKII cells are a well established and well-characterised epithelial cell model, given the experienced technical difficulties in working with this canine cell line it is worth considering other epithelial cell models. A significant disadvantage of changing cell model is that many of the planned experimental techniques have been established in MDCK cells and may not be easily applied to all epithelial cells. Also, given the MDCK cell line is currently unique in the observation of cell-cell contact FAM staining by immunofluorescence, the study of FAM in this cell line may offer insights into the role of FAM in cell-cell adhesion that would not be obvious in other model systems.

One key advantage to using an alternative cell line, is that it simplifies the observation of endogenous proteins. Most antibodies are not raised against canine protein homologues, and do not recognise the canine proteins as efficiently as the human or murine homologues against which they were raised. This was found to be particularly true of the N1 anti-FAM antibody. This antibody was generated against the first 21aa of murine FAM (N21), which is identical to the corresponding human sequence but is now known to differ from the canine sequence by a single amino acid substitution. Canine FAM has an N15→S substitution and although asparagine and serine have weakly similar chemical properties empirical evidence demonstrates this substitution induces a sufficient structural change to inhibit the recognition of canine FAM by polyclonal Abs raised against the murine peptide. This single amino acid substitution between murine and canine FAM is unfortunate given the high degree of identity between these two proteins (97.6% when murine aa959-1094, which are not present in the NCBI canine protein sequence, are excluded from the analysis). To examine endogenous canine FAM, antibodies against canine N21-FAM peptide should be effective for use in immunofluorescence.

## ***Conclusion***

Gain of function analysis of the role of FAM in polarised epithelia has been approached by attempts to generate unconditionally or inducibly expressing stable MDCKII cell lines. Unfortunately, these attempts have not been fruitful, but many alternative methodologies remain to be applied to the study of FAM in this model epithelial system. It should also be noted that while analysis of total FAM levels in MDCKII cell lines was hampered by an initial inability to reliably detect canine FAM protein, the recent release of canine FAM nucleic acid and protein sequence information will be of great use in designing tools for future application in the study of FAM.

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