The Role of Deubiquitylating Enzymes in Cell-Cell Adhesion and Development

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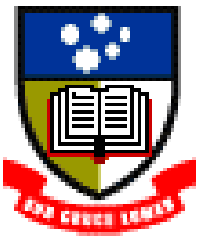

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Abstract

Ubiquitylation is a versatile post-translational modification that participates in regulation of protein stability, via proteasomal and lysosomal degradative pathways, regulation of membrane protein internalisation and other trafficking events, and regulating the biological activity of some proteins independent of degradation. The diverse functions of ubiquitylation as a post-translational protein modification allow speculation that regulation of protein ubiquitylation status may be of crucial importance during the dynamic process of development. A screen of known, and suspected, ubiquitin pathway enzymes was designed to test this hypothesis. Whole mount *in situ* hybridisation was conducted on early post implantation mouse embryos to determine expression patterns of the ubiquitin pathway enzyme targets. This screen was not pursued in depth due to difficulties in resolving doubts regarding the sensitivity of the method and the validity of weak ubiquitous staining patterns.

The FAM deubiquitylating enzyme is a known developmentally regulated ubiquitin pathway enzyme, and although believed to antagonise the conjugation of ubiquitin to specific substrates its cell biology remains poorly characterised. In different cellular contexts FAM has been reported to localise to points of cell-cell contact or to endosomes, and circumstantial evidence suggests a role in regulating trafficking of a cell-cell adhesion complex (Murray et al., 2004; Taya et al., 1999; Taya et al., 1998). It was sought to further investigate the role of FAM in cell-cell adhesion in the well characterized polarized epithelial cell line, MDCKII, by creating clonal MDCKII cell lines that overexpress FAM. These cell lines were to be analysed for alterations in cell-cell adhesive properties and the biochemistry of proposed FAM substrates, which include the cell adhesion molecules β-catenin, E-cadherin and AF-6. MDCKII cell lines expressing exogenous V5-tagged murine FAM were successfully isolated, but failed to show changes in cell-cell adhesive properties. Generation of an antibody that reliably recognised both the canine and murine FAM protein demonstrated that the FAM-V5 expressing cell lines did not have increased total FAM protein. Other approaches taken to facilitate the study of FAM include attempts to express GFP-FAM fusion proteins and to generate an inducible FAM overexpressing cell line. Further alternative approaches are discussed.

Declaration of Originality

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Susan M Millard

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I specifically acknowledge the contributions of the following people to this study. Michaela Scherer performed the Western analysis presented in Figure 4.2B and began the process of making pEF-DEST51-FAM stable cell lines in HEK293T cells, described in Chapter 4. I had the pleasure of supervising Jonathon Webb for a third year research project in which he performed the biotin amplified immunofluorescence presented in Figure 5.2. Suzanne Bresatz performed the tyramide signal amplification immunofluorescence presented in Figure 5.4. Stephen Wood gave generously of his time in the attempt to generate a FAM inducible expression system in MDCKII cells using the T-REx system, as described in Chapter 6. Stephen performed the transfection, selection, clonal expansion and Northern blot screening of the parental cell line, and the selection and clonal expansion of the T-REx-FAM clones. Western analysis and quantitative real-time PCR analysis of these clones, presented in Figures 6.12 and 6.13, was performed by Lachlan Jolly.

I would also like to acknowledge those organisations that provided financial support and bench space throughout my PhD. My personal finances were generously supported for three and a half years by an Australian Postgraduate Award and a Premier's Biosciences Scholarship, awarded through the University of Adelaide. The Centre for the Molecular Genetics of Development (CMGD) provided much appreciated support for conducting the experiments detailed in this thesis. I begun my PhD as an on campus member of the Discipline of Biochemistry within the University of Adelaide, but later moved off campus

to CHRI (Child Health Research Institute), where work described in this thesis regarding the deubiquitylating enzyme FAM was undertaken.

I also wish to make a number of special mentions for those who have supported me through my long, arduous writing up phase. I'm very grateful to Dr Gary Leong for offering me employment and understanding while I was yet to finish my thesis. His willingness to be flexible regarding my work hours and to proofread many of the following chapters has been greatly appreciated. To all my family, thanks for your love and for not asking to often "When's your thesis going to be finished?" And finally, I'd like to thank the most amazing woman in my life, Annette Shewan. Her love, support and faith in me has been my greatest source of motivation. This thesis has been a long time in the writing, but it's finally finished, and it's finally time to take a holiday together.

Abbreviations

aa	amino acid
AF-6	*ALL-1* fusion partner from chromosome 6
AIP4	atrophin-1-interacting protein 4
AJ	adherens junction
AP	adaptor protein complex
APC	adenomatous polyposis coli tumor suppressor protein
βME	β-mercaptoethanol
bp	base pair
BSA	bovine serum albumin
°C	degrees Celsius
cDNA	complimentary deoxyribonucleic acid
CLIM	cofactor of LIM homeodomain proteins
CPS	carboxypeptidase S
CXCR4	CXC chemokine receptor4
DCX	doublecortin
DUB	deubiquitylating enzyme
E1	ubiquitin activating enzyme
E2	ubiquitin conjugating enzyme
E3	ubiquitin ligase
E4	ubiquitin chain elongating factor
E6AP	E6 associated protein
ECF	enhanced chemifluorescence
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
Eph	ephrin
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EGTA	ethyleneglycol-bis (β-aminoethyl ether)-*N,N'*-tetraacetic acid
ENTH	epsin NH2-terminal homology
EPL	early primitive ectoderm like
Eps15	EGFR pathway substrate clone no15
Epsin	Eps15 interacting protein
ER	endoplasmic reticulum
ES	embryonic stem
ESCRT	endosomal sorting complex required for transport
Faf	fat facets
FAM	fat facets in mouse (also used to refer to vertebrate homologues of FAM)
FAMCAT	region spanning murine FAM's catalytic domain (aa1475-1918)
FCS	foetal calf serum
GGA	Golgi-localised, γ-ear containing, ARF binding protein

GHR	growth hormone receptor
gm	gram
GPCR	G-protein coupled receptor
GSK3β	glycogen synthase kinase 3β
GST	glutathione S-transferase
HA	haemaglutinin epitope tag
HECT	homology to E6AP C-terminus
HEF-1	human enhancer of filamentation 1
HEK293T	human embryonic kidney 293T
Hepes	*N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulphonic acid
HRP	horse radish peroxidase
Hrs	hepatocyte growth factor-regulated tyrosine kinase substrate
IκBα	inhibitor of nuclear factor κB-α
IPTG	isopropyl-β-D-thiogalactoside
JAM	junctional adhesion molecule
kD	kilodalton
l	litre
LEF	lymphoid enhancer factor-1
Lqf	Liquid facets (*Drosophila* epsin1)
m	metre
M	molar concentration
μM	micro-molar concentration
MAPK	mitogen activated protein kinase
MDCKII	Madin-Darby canine kidney, strain II
Mdm2	murine double minute 2
min	minutes
mUb	monoubiquitin
MVB	multivesicular body
NEDD	neurally expressed, developmentally down-regulated
NFκB	nuclear factor κB
nM	nano-molar concentration
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
PLZF	promyelocytic leukemia Zn(2)+ finger protein
PMSF	phenylmethylsulphonyl fluoride
pUb	polyubiquitin
RING	really interesting new gene
RLIM	RING finger LIM domain binding protein
RTK	receptor tyrosine kinase

$SCF^{\beta\text{-TrCP}}$	Skp, Cullin, F-box ubiquitin ligase complex, where the F-box protein is β-TrCP
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
Siah	seven in absentia homolog
SUMO	small ubiquitin-related modifier
TCF	T-cell factor
TER	Transepithelial Electrical Resistance
TGN	*trans*-Golgi network
TJ	tight junction
TNFα	tumour necrosis factor α
Trx	thioredoxin
TSA	Tyramide Signal Amplification
Tsg101	tumour susceptibility gene 101
TTBS	Tris buffered saline + Tween-20
Ub	ubiquitin
UBA	ubiquitin associated
Ubl	ubiquitin-like domain
UBP	ubiquitin specific processing proteases
UEV	ubiquitin E2 variant
UIM	ubiquitin interacting motif
USP	ubiquitin specific processing proteases
UCH	ubiquitin C-terminal hydroxylase
ZO-1	zonula occludens protein 1

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