FUNCTIONAL ANALYSIS OF THE DEUBIQUITYLATING ENZYME FAT FACETS IN MOUSE IN PROTEIN TRAFFICKING

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Abstract

Fat facets in Mouse (FAM) or mUSP9x is a deubiquitylating enzyme of the USP class. Knockdown of FAM protein levels in mouse pre-implantation embryos by antisense oligonucleotides is known to prevent embryos from progressing to the blastocyst stage indicating an important role for FAM in early mammalian development. In mammals, the *Fam* gene is located on the X-chromosome. In mice, the Y homologue, *Dffry* or *usp9y*, is expressed exclusively in the testes and maps to the Sxr^b deletion (Brown et al., 1998). Sxr^b is associated with an early post-natal blockage of spermatogonial proliferation and differentiation leading to absence of germ cells (Bishop et al., 1988; Mardon et al., 1989). The human Y homologue of *Fam* is closely associated with oligozoospermia (Sargent et al., 1999; Sun et al., 1999) and the human X homologue has been linked to the failure of oocytes to pass through the first meitoc prophase in Turner syndrome (Cockwell et al., 1991; Speed, 1986) Despite these associations, the substrates and precise role of *Fam* and its homologues in these processes have not yet been defined. Due to the complex nature of *Fam* expression and the lack of data tying FAM to specific cellular functions, much attention has been paid in identifying interacting partners and cellular targets of FAM activity to aid in the definition of its role in the cell and development.

Three common molecular biology techniques were applied here in an attempt to further characterise known interactions of FAM, including interactions with the cell adhesion molecule β-catenin and the protein trafficking pathway proteins epsin-1 and itch. The aim of these investigations was to generate FAM mutants that could abolish individual interactions, enabling investigation of individual interactions in cellular function and development. These experiments failed to identify the amino acids of FAM that were critical for its interactions with β-catenin, epsin-1, or itch. Experiments aimed at characterising a novel ubiquitin-like domain located in the N-terminal half of the FAM protein, did however identify novel interactions of FAM with the three Golgi associated adaptor proteins GGA1, GGA2, and GGA3. Further investigations prompted by this interaction, examined the role of FAM in the trafficking of proteins from the Golgi apparatus. Cellular FAM protein levels were altered either by exogenous expression of FAM protein or knockdown of endogenous FAM using FAM specific shRNA triggers. The cellular protein levels and extent of post-translational modification of eleven lysosomal proteins were monitored in each case. It was found that increased FAM protein levels resulted in decreased cellular protein levels of five of the eleven lysosomal proteins studied. In contrast, a reduction in FAM protein levels was found to result

in an increase in the cellular protein levels of eight of the eleven lysosomal proteins. This study provides the first evidence of a deubiquitylating enzyme that is able to interact with the GGA proteins. It is also the first to describe a deubiquitylating enzyme that can affect the biosynthesis of lysosomal proteins and provides valuable new insight into the cellular function of FAM/USP9X.

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 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.

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Abbreviations:

4S	4-iduronate-sulphatase
α	anti
AC-LL	acidic di-leucine motif
AF-6	*ALL-I* fusion partner from chromosome 6
a-Gal	α-galactosidase
α-Gal	α-galactosidase
AP	adaptor protein complex
ASA	aryl sulphatase A
ASM	acid sphingomyelinase
ATP	adenosine tri-phosphate
b-Gal	β-galactosidase
β-Gal	β-galactosidase
bp	base pair
BSA	bovine serum albumen
ºC	degrees Celsius
cbl	Casitas B-lineage lymphoma
CCV	clathrin coated vesicle
CD-MPR	cation dependent mannose-6-phosphate receptor
CI-MPR	cation-independent mannose -6-phosphate receptor
Dcx	doublecortin
DMEM	Dulbeco's Modified Eagle Medium
Doa4	dead on arrival 4
DUB	deubiquitylating enzyme
E1	ubiquitin activating enzyme
E2	ubiquitin conjugating enzyme
E3	ubiquitin ligase
E4	ubiquitin chain elongating factor
ECL	enhanced chemiluminescence

EGFP	enhanced green fluorescent protein
E. coli	*Escherichia coli*
EDTA	ethylene diamine tetra acetic acid
EE	early endosome
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ENTH	epsin NH2-terminal homology
Eps15	EGFR pathway substrate clone no 15
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)
FAM CAT	region spanning murine FAM's catalytic domain (amino acids 1475-1918)
FBR	FAM-binding region
FCS	foetal calf serum
FD	FAM domain
GAA	acid alpha glucosidase
GAE	gamma adaptin ear-like
GAL4-DBD	DNA binding domain of the GAL4 transcription factor
GAL4-AD	activation domain of the GAL4 transcription factor
GAT	GGA and Tom-1
GFP	green fluorescent protein
GGA	Golgi-localised, γ-ear containing, ARF binding protein
gm	gram
GPC	Golgi to plasma membrane carrier
GTP	guanidine tri-phosphate
GSK3β	glycogen synthase kinase 3β
GST	glutathione S-transferase
GW	gateway

HA	haemaglutinin epitope tag
HECT	homology to E6AP C-terminus
HEK 293T	human embryonic kidney 293T
hr	hour/hours
HRP	horse radish peroxidase
Hrs	hepatocyte growth factor-regulated tyrosine kinase substrate
HSC70	heat shock cognate protein 70
I2S	iduronate-2-sulphatase
IdUA	Iduronidase
IκB	inhibitor of nuclear factor κB
IκBK	inhibitor of nuclear factor κB kinase
IPTG	isopropyl-b-D-thiogalactopyranoside
kb	Kilobase pairs
kDa	KiloDalton
L	Litre
LAMP	lysosomal membrane associated protein
LB	luria broth
LE	late endosome
LEF	lymphoid enhancer factor
LIMP	lysosomal integral membrane protein
Lqf	Liquid facets
M	molar concentration
M6P	mannose-6-phosphate
M6PR	mannose-6-phosphate receptor
MDa	mega Dalton
mM	milli-molar concentration
μM	micro-molar concentration
min	minute(s)
MVB	multivesicular body
Na^+	ionic sodium

NaCl	sodium chloride
NF-κB	nuclear factor κB
nM	nano-molar concentration
μL	micro-litres
μg	micro-grams
N-sulph	N-sulphatase
OD	optical density
orf	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBST	phosphate buffered saline with 0.3% Tween 20
PBSTM	phosphate buffered saline with 0.3% Tween 20 and powdered milk (Diploma)
PCR	polymerase chain reaction
PEST	praline (P), glutamic acid (E), serine (S), and threonine (T) – an amino acid sequence associated with rapid protein degradation
PMSF	phenyl methyl sulphonyl chloride
PVC	pre-vacuolar compartment
Rcf	relative centrifugal force
RING	really interesting new gene
RTK	receptor tyrosine kinase
SDS	sodium dodecyl sulphate
sec	second(s)
Sap-C	saposin-C
STAM	signal transducing adaptor molecule
SUMO	small ubiquitin-related modifier
TCF	T-cell factor
TGN	*trans*-Golgi network
TGF	transforming growth factor
TEMED	N,N,N’N’-tetramethyl-ethenediamine
TKB	tyrosine kinase binding domain

TTBS	tris buffered saline with Tween 20
Ub	ubiquitin
UBA	ubiquitin associated
UBL	ubiquitin-like
UBLD	ubiquitin-like domain
UDP	ubiquitin domain protein
UBP	ubiquitin specific processing proteases
UEV	ubiquitin E2 variant
UIM	ubiquitin interacting motif
USP	ubiquitin specific processing proteases
UCH	ubiquitin C-terminal hydroxylase
V	Volts
VHS	Vps27/Hrs/Stam
Vol/vol	volume per volume
Vps	vacuolar protein sorting factor

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