The Genetics of 3-M Syndrome: Unravelling a Potential New Regulatory Growth Pathway

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Key Words

Growth disorder • 3-M syndrome • Silver-Russell syndrome • Ubiquitination • Insulin like growth factor signalling

Abstract

3-M syndrome is an autosomal recessive primordial growth disorder characterised by severe postnatal growth restriction caused by mutations in *CUL7*, *OBSL1* or *CCDC8*. Clinical characteristics include dysmorphic facial features and fleshy prominent heels with a variable degree of radiological abnormalities. *CUL7* is a structural protein central to the formation of an ubiquitin E3 ligase that is known to target insulin receptor substrate 1 for degradation. *CUL7* also binds to p53 and may be involved in the control of p53-dependent apoptosis. *OBSL1* is a cytoskeletal adaptor protein that was thought to play a central role in myocyte remodelling, and *CCDC8* has no defined function as yet. However, the physical interaction of *OBSL1* with both *CUL7* and *CCDC8* and its potential role in the regulation of *CUL7* expression suggest all three proteins are members of the same growth-regulatory pathway. Future work should be directed to investigating the function of the 3-M syndrome pathway and in particular the role in the insulin like growth factor I signalling pathway with a view of potentially revealing new therapeutic targets and identifying key regulators of cellular growth.

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Background: 3-M Syndrome and Differential Diagnosis

The characterisation of disorders associated with extreme pre- and postnatal growth restriction allows the identification of novel human growth regulators. This has been demonstrated by studies of microcephalic osteodysplastic primordial dwarfism type II with the identification of mutations in pericentrin (*PCNT*), signifying the importance to human growth of the mitotic spindle in regulating normal chromosome segregation [1].

Through the study of molecular defects of the primordial growth disorder 3-M syndrome (MIM 273750 and 612921), we have identified a novel pathway controlling human growth. 3-M syndrome is a distinct growth disorder first described by Miller, McKusick and Malvaux in 1975, with the name derived from the shared initial letter of the authors' surnames [2]. 3-M syndrome is characterised by prenatal growth restriction in the absence of recognisable maternal or placental pathology and by the failure of postnatal catch-up growth resulting in significant proportionate short stature without associated microcephaly often seen in microcephalic osteodysplastic primordial dwarfism type II.

Silver-Russell syndrome (SRS), a severe short stature condition with a high degree of phenotypic overlap with

Table 1. Differential diagnosis between 3-M syndrome and SRS – a comparison of the typical clinical features [4–16]

3-M syndrome	SRS
IUGR mean BW –3 SD	Asymmetric IUGR mean BW –3.7 SD, mean BL –4 SD
Adult height in the range of –8 to –4 SD	Mean adult height of –4.2 SD
Triangular face, full fleshy lips, frontal bossing, upturned fleshy nose	Triangular face, thin lips, frontal bossing
Tall vertebral body, slender long bones	Limb asymmetry
Normal intelligence	Variable incidence of learning difficulties
Prominent heels	Normal heels
Autosomal recessive	Genetically heterogeneous
~65% <i>CUL7</i> mutations	~50% 11p15 hypomethylation
~30% <i>OBSL1</i> mutations	~10% maternal uniparental disomy of chromosome 7
~5% <i>CCDC8</i> mutations	~40% unknown cause

BW = Birth weight; BL = birth length.

3-M syndrome, should be considered in the differential diagnosis. There have been over 400 reported cases of SRS [3]. The key diagnostic characteristics of SRS include a birth weight ≤ -2 standard deviation score (SDS), postnatal growth restriction, normal head circumference, triangular-shaped face with a broad forehead and a pointed, small chin with a wide, thin mouth and body asymmetry [4]. Patton [5] proposed that the slender long bones and tall vertebrae in 3-M syndrome are important in distinguishing between the two conditions. However, many 3-M syndrome patients do not show these features, suggesting that this should probably not be used for differential diagnosis. Learning difficulties can be a part of SRS but are not seen in 3-M syndrome, whereas prominent heels, a fleshy protrusion on the back of the heel, are an almost universal feature in 3-M syndrome patients but not in SRS patients (table 1).

Genetics of Silver-Russell Syndrome

SRS is genetically heterogeneous and often sporadic [17], and around 60% of cases are associated with genetic abnormalities: approximately 50% have imprinting abnormalities on chromosome 11p15, while approximately 10% demonstrate uniparental disomy of chromosome 7 (UPD7) [18].

Hypomethylation of the insulin like growth factor II (*IGF-II*) and *H19* imprinting centre at 11p15 has recently been associated with SRS [19]. While loss of DNA methylation of this site results in an SRS phenotype, hypermethylation of the same site results in the overgrowth syndrome Beckwith-Wiedemann [19]. Eggermann et al.

[20], Gicquel et al. [21] and Eggermann [22] showed that a number of patients exhibiting an SRS phenotype have a maternal 11p15 duplication that leads to loss of *IGF-II* expression and concomitant short stature.

UPD7 involves maternal chromosome regions 7p12-p14 and 7q31-qter [23]. The region at chromosome 7p12-p14 contains a number of imprinted candidate genes including *GRB10*, *IGFBP1* and *IGFBP3*. Mutation analysis of *GRB10*, however, has not revealed any pathogenic variants in SRS patients [3]. The causative gene in the region 7q31-qter has likewise not been identified [17].

Clinical Features of 3-M Syndrome

The main clinical features of 3-M syndrome are severe postnatal growth restriction with normal but recognisable facial features including triangular face, flat maxillae and prominent forehead. Other facial features include a fleshy tipped nose, pointed chin and long philtrum [2, 13, 14]. Skeletal abnormalities including relatively tall vertebrae and slender long bones are seen with variable frequency [13, 24, 25]. Other features commonly associated with 3-M syndrome are a short neck, winged scapulae, short thorax, transverse chest groove, hyperlordosis, developmental dysplasia hip and spina bifida occulta [2, 8, 12–15, 24–26]. In younger patients, prominent heels are an almost universal feature, particularly in those with a genetic diagnosis. Unlike many other short stature conditions, 3-M syndrome is associated with normal intelligence and developmental milestones are achieved. Adult height is in the range of 115 to 150 cm (–8 to –4 SD) (fig. 1) [10].

Fig. 1. Clinical features of 3-M syndrome individuals. **a** 3-M syndrome patient with a *CCDC8* mutation. **b, c** Two 3-M syndrome families in which *CUL7* mutations have been found. **d** A 3-M syndrome patient with an *OBSL1* mutation. All patients have facial

dysmorphism. Typical features include a triangular face, fleshy tipped nose, frontal bossing, midfacial hypoplasia and a pointed chin. Prominent heels are particularly evident in younger 3-M syndrome patients.

Genetics of 3-M Syndrome

The initial 3-M syndrome locus was identified on 6p21.1, and subsequent candidate analysis of the region revealed that mutations in the cullin 7 gene (*CUL7*) cause 3-M syndrome [10]. As part of that investigation, we identified a smaller subset of patients who did not carry *CUL7* mutations but appeared to present with an identical phenotype (fig. 1). Further autozygosity mapping revealed a second locus located on 2q35 with mutations in the gene encoding for obscurin-like 1 (*OBSL1*) found to be the underlying cause [24]. In addition, a number of patients have neither *CUL7* nor *OBSL1* mutations, and exome sequencing in 3 such patients revealed that mutations in the coiled-coil domain containing protein 8 (*CCDC8*) cause the 3-M syndrome [27].

CUL7 Mutations Cause 3-M Syndrome

Huber et al. [10] performed autozygosity mapping with seven consanguineous 3-M syndrome families of Tunisian, Moroccan and French descent. This revealed a 3.84-Mb homozygous region shared by all seven families on chromosome 6p21.1. In addition, all four Tunisian families shared a 0.47-Mb region of common haplotype indicating the location of the candidate gene. Subsequent analysis revealed pathogenic mutations within *CUL7*. *CUL7* mutations are found throughout the whole gene, although approximately half of the mutations are located in the cullin domain. The majority of *CUL7* mutations cause premature termination and are therefore likely to lead to complete loss of the *CUL7* protein; however, a small number are missense mutations and thus predicted to lead to the generation of a nonfunctional protein product (fig. 2) [10, 25, 28].

3-M syndrome shares a high degree of similarity with two other short stature conditions: gloomy face syn-

Fig. 2. Gene layout of *CUL7*. Intron and exon structure of *CUL7* (non-coding UTRs are indicated by grey shading) with arrows indicating the location of known mutations and the location of the 3 main functional domains of *CUL7* protein are shown.

drome [26] and the recently identified Yakut short stature syndrome [25]. Both have the same genetic cause as 3-M syndrome and are therefore unlikely to be separate entities, but rather to be part of a wider 3-M syndrome spectrum. Huber et al. [10] suggested the inclusion of gloomy face into 3-M syndrome following similarities in long-term follow-up and, most importantly, the identification of mutations in *CUL7* in both sets of patient. Maksimova et al. [25] reported that the Yakut short stature syndrome patients suffered variable neonatal respiratory distress that had not been previously recognised as a feature of 3-M syndrome. However, other clinical features and characteristics of 3-M syndrome and identification of mutations in *CUL7* would suggest that the Yakut short stature syndrome patients actually have 3-M syndrome.

CUL7 and the Ubiquitin-Proteasome Pathway

The protein product *CUL7* is the most recently defined member of the cullin family and is an integral part of the ubiquitin-proteasome pathway acting as a structural protein in the formation of a SKP1-*CUL7*-FBXW8-ROC1 (SCF) E3 ubiquitin ligase complex [29].

The ubiquitin-proteasome pathway is a major protein degradation pathway in which proteins are targeted for degradation, a process which involves attachment of ubiquitin to a target protein by the E3 ubiquitin ligase complex followed by degradation by the 26S proteasome. The roles of SCF complexes and the ubiquitination system have been reviewed previously [30].

Unlike other cullin proteins which can interact with several F-box proteins to form alternative SCF complexes, FBXW8 is currently the only F-box protein known to interact with *CUL7* (fig. 3). In keeping with the clinical feature of 3-M syndrome, targeted disruption of *Cul7* in mice resulted in intrauterine growth restriction (IUGR) and placental abnormalities. Arai et al. [31] showed that *Cul7*^{-/-} embryos survived to embryonic day 18.5 but did not survive birth. In addition to the severe growth restriction seen, *Cul7*^{-/-} embryos were unable to breathe unaided, and it was shown that the lungs had markedly reduced alveolar space compared to wild type [31]. Although a 3-M syndrome foetus with a *CUL7* mutation had no obvious alveolar abnormalities, there was insufficient development of the cartilaginous lamina, which may explain the respiratory distress that was seen in a subset of the Yakut 3-M syndrome patients [25]. The *Cul7*^{-/-} phenotype is similar to that exhibited by *Fbxw8*^{-/-} mice with severe growth restriction and some respiratory failure [32]. *Fbxw8*^{-/-} mice have a reduced placental size but appear to have no isolated placental abnormalities. Tsutsumi et al. [33] confirmed the *Fbxw8*^{-/-} phenotype and underlined the similarities between *Fbxw8*^{-/-} and *Cul7*^{-/-} mice. Growth restriction of *Fbxw8*^{-/-} mice continued postnatally, but the mice were otherwise normal. Arai et al. [31] established that Fbxw8 protein levels were reduced in *Cul7*^{-/-} cells, with Tsutsumi et al. [33] demonstrating that *Cul7* levels were also reduced in *Fbxw8*^{-/-} mouse embryonic fibroblast (MEF) cells. *Fbxw8*^{-/-} MEFs grew very poorly when compared to *Fbxw8*^{+/+} or *Fbxw8*^{+/-} MEFs. The increased severity in the

Fig. 3. CUL7 SCF complex. Schematic representation of the CUL7 SCF complex (E3 core in red) showing polyubiquitination of a substrate protein, which is then targeted for degradation by the 26S proteasome leaving free ubiquitin and short peptides.

phenotype of mice which lack *Cul7* compared to *Fbxw8* is especially evident as 30% of *Fbxw8*^{-/-} mice survive birth. This would suggest that Cul7 has further cellular functions that are not shared with Fbxw8 [32, 34].

CUL7 Interaction Network

To date, there is limited data regarding the known proteolytic targets of the CUL7 SCF complex. Two studies have shown the F-box component of the CUL7 SCF complex, FBXW8 interacts with and targets the degradation of cyclin D1 [35] and insulin receptor substrate 1 (IRS-1) [36].

Cyclin D1

The role of cyclin D1 as an important regulator of cell cycle progression and its role in cancer due to aberrant expression have been previously reviewed [37]. Okabe et al. [35] demonstrated that cyclin D1 associates with FBXW8 – an interaction dependent on MAPK-mediated T286 phosphorylation suggesting that the CUL7 SCF complex is responsible for cyclin D1 ubiquitination. However, further evidence that multiple SCF complexes are responsible for cyclin D1 ubiquitination and subsequent degradation makes it difficult to determine the importance of FBXW8/CUL7-mediated cyclin D1 degradation to cell cycle regulation [38].

IRS-1

IRS-1 is a member of a family of proteins that act as adaptor proteins in both the insulin and IGF-I signalling cascades and are involved in numerous biological pathways [39]. In 2008, Xu et al. [36] performed a proteomic analysis to discover potential targets of the CUL7 SCF complex by using a stable FBXW8-expressing cell line to immunoprecipitate interacting partners and thus probable substrate proteins. This led to the identification of IRS-1 as a proteolytic target of the CUL7 SCF complex [36]. IRS-1 binds to the insulin or IGF-I receptors and is phosphorylated at the tyrosine-rich C-terminal region. SH-2 domain-containing proteins are then recruited, leading to the activation of downstream pathways including AKT and MAPK/ERK [39]. Xu et al. [36] demonstrated that IRS-1 was polyubiquitinated in the presence of FBXW8, and this ubiquitination led to increased degradation of the IRS-1 protein. Using a mouse knockout model of *Cul7*, they showed in MEF cells from *Cul7*^{-/-} mice that although the protein levels of Irs-1 increased dramatically in *Cul7*^{-/-} MEFs, mRNA expression of *Irs-1* was not significantly altered between *Cul7*^{-/-} and *Cul7*^{+/+} cells. In addition to the increased levels of Irs-1, *Cul7*^{-/-} MEFs also grew very poorly when compared to *Cul7*^{+/+} or *Cul7*^{+/-} MEFs, and the growth rate of the *Cul7*^{-/-} MEFs decreased over time. At passage 7, *Cul7*^{-/-} MEFs became flat and enlarged in comparison to *Cul7*^{+/+} MEFs. This indicated that *Cul7* knockout may induce cellular senescence [36]. It is possible that accumulation

Fig. 4. Gene layout of *OBSL1*. Intron and exon structure of *OBSL1* (non-coding UTRs are indicated by grey shading) with arrows indicating the location of known mutations clustered near the N-terminal region, thus affecting all 3 protein isoforms. The three isoforms arise from use of alternative stop codons in exons 10, 16 and 22 and encode for proteins with variable numbers of C-terminal Ig domains.

of IRS-1 may activate CUL7-mediated cellular senescence, which may in part explain the growth restriction seen in 3-M syndrome patients [36].

Non-Proteolytic Function of CUL7

Atypical of other cullin proteins, CUL7 also contains a CPH domain (a conserved region found in CUL7/PARC/HERC2) [40]. This domain is responsible for some non-proteolytic functions of CUL7, including acting as a p53 binding region and, although the CUL7 E3 ligase is not responsible for the degradation of p53, it may participate in its activation and cellular localisation [40–43]. Whether CUL7 contributes to p53-dependent apoptosis remains to be fully determined; however, Kim et al. [44] demonstrated that CUL7 expression prevents c-Myc- and N-Myc-mediated apoptosis in a p53-dependent manner.

OBSL1 Mutations Cause 3-M Syndrome

In a large cohort of 3-M syndrome patients who did not carry *CUL7* mutations, we performed autozygosity mapping revealing a second 3-M syndrome locus located at chromosome 2q35-q36.1, spanning 5.7 Mb. Further haplotype analysis refined the critical region to a 1.27-Mb region. Pathogenic mutations were subsequently

identified in the *OBSL1* gene, of which all were presumptive null mutations [24]. Huber et al. [45] also identified *OBSL1* mutations in a separate cohort of 3-M syndrome patients, including the identification of the first *OBSL1* missense mutation (fig. 4).

To date, approximately 65% of mutations described in 3-M syndrome patients are found in *CUL7*, with 30% identified in *OBSL1* [10, 24, 25, 28, 45].

OBSL1 Structure and Function

OBSL1 is related to the giant muscle protein obscurin [46]. Gene duplication and rearrangement of obscurin resulted in the formation of two genes: *SPEG* (striated muscle preferentially expressed gene) and *OBSL1*. *SPEG* was first identified as a serine/threonine kinase with isoforms expressed in smooth, skeletal and heart muscle as well as brain tissue [47]. It was subsequently found that *SPEG* shared its arrangement of these kinase domains with obscurin [48]. *OBSL1* was identified by Geisler et al. [46] and is located within 100 kb of *SPEG* on chromosome 2q35. The fibronectin domain of *OBSL1* is most closely related to the fibronectin domain at the amino-terminal of obscurin [46]. Previously, Sutter et al. [48] showed that the carboxy fibronectin domain of obscurin closely resembled the fibronectin domain of *SPEG*. Also, *SPEG* is transcribed by the forward strand of chromo-

some 2, whereas *OBSL1* is transcribed by the reverse strand. Therefore, the duplication event of obscurin that gave rise to *OBSL1* and *SPEG* also resulted in a genomic rearrangement.

OBSL1 exists as three groups of isoforms A, B and C that share a common translational start site which is also conserved between human, mouse and rat [46]. The variability of the three *OBSL1* isoforms is derived from the use of alternative stop codons. Geisler et al. [46] identified a number of different transcripts ranging in size from 3.3 to 5.9 kb. All *OBSL1* isoforms share a common amino-terminal region consisting of four Ig domains and a fibronectin domain. The smaller transcripts encode three carboxy-terminal Ig domains, whereas the longest known transcript of isoform A encodes thirteen carboxy-terminal Ig domains [46]. Interestingly, Fukuzawa et al. [49] have suggested that further differentially spliced variants of *OBSL1* exist. Three human expressed sequence tags have been identified in which the first coding exon is missing (DA680669, BX381924 and DA427488), suggesting the possible use of one of two alternative translational start sites located in exon 3, both in the same reading frame as the original translational start site. The potential new alternative translational start site residues would correspond to p.M413 and p.M479 of the current *OBSL1* isoforms.

Cellular localisation studies carried out in adult rat cardiac myocytes revealed that *OBSL1* is found at the intercalated discs, the perinuclear region and the Z lines with low level expression at the M bands [46]. During remodelling of myocytes to form new sarcomeres, *OBSL1* localises to the perinuclear region but dissociates from both the Z lines and M bands [46]. Geisler et al. [46] also suggested that *OBSL1* remains associated with the nuclear envelope and surrounding cytoskeletal network, but that this is dependent on the morphology of the nucleus.

OBSL1 is composed entirely of Ig-like domains with a single fibronectin domain. These domains have been previously identified in titin, myomesin and obscurin in which they play a role in protein-protein interaction [46]. Due to the cellular localisation in cardiac myocytes and the predicted domain structure of *OBSL1*, Geisler et al. [46] postulated that *OBSL1* may function as a cytoskeletal adaptor protein linking membrane-bound nuclear proteins to the cytoplasmic support network. Since Geisler et al. [46] also found that *OBSL1* was expressed in a wide variety of cell types, it is possible that *OBSL1* may have a broader role as a scaffolding protein. During the investigation by Fukuzawa et al. [49], the N-terminal

region of obscurin was found to interact with both titin and myomesin; at the same time, interaction between these two proteins and *OBSL1* was also established. Similar to obscurin, it was found that the N-terminal region of *OBSL1*, which is homologous to the N-terminal region of obscurin, is also responsible for the interaction with titin and myomesin [49–51]. Fukuzawa et al. [49] assumed that since *OBSL1* lacks any of the signalling domains of obscurin, its primary role would be as a structural protein.

Both Geisler et al. [46] and Fukuzawa et al. [49] postulated that the involvement of *OBSL1* as a key component of the cytoskeletal network indicated a role in the progression of myopathies. Fukuzawa et al. [49] suggested that this would be due to loss of titin interaction at the M lines of sarcomeres. However, our recent report identifying *OBSL1* mutations as a cause of 3-M syndrome indicated a broader role for *OBSL1* in the CUL7 ubiquitination pathway [24]. Currently, there is only rudimentary knowledge of *OBSL1* and it is clear that further characterisation is required to elucidate the function of *OBSL1* and specifically its impact on the regulation of human growth. To date, we did not identify muscle weakness as a feature of 3-M syndrome.

CCDC8 Mutations Cause 3-M Syndrome

We and others have reported some cases of 3-M syndrome patients who have neither *CUL7* nor *OBSL1* mutations, suggesting involvement of further genes [24, 45]. We recently identified mutations in *CCDC8* in a small cohort of 3-M syndrome patients. Two different mutations have so far been identified in five unrelated families and both result in the generation of a premature stop codon and subsequent loss of function. We demonstrated by immunoblotting that a 3-M syndrome patient with a *CCDC8* mutation had no detectable *CCDC8* protein and that 3-M syndrome patients with either *CUL7* or *OBSL1* mutations had normal levels of *CCDC8* protein [27].

Elucidating the 3-M Syndrome Pathway

Thus, three apparently unrelated cytoplasmic proteins have been implicated in the pathogenesis of the human growth disorder 3-M syndrome: one an ubiquitin ligase (*CUL7*), one a cytoskeletal adaptor (*OBSL1*) and one a protein of undefined function (*CCDC8*). In the current

literature, 94 families have a genetic confirmation of 3-M syndrome: 63 families have *CUL7* mutations, 26 *OBSL1* mutations and 5 *CCDC8* mutations. We have previously shown that loss of *OBSL1* by siRNA also leads to reduction of *CUL7* expression, suggesting the two proteins may be linked [24]. However, it appears that the loss of either *CUL7* or *OBSL1* in patient-derived cells does not affect *CCDC8* levels. Co-immunoprecipitation assays revealed that *CCDC8* and *OBSL1* physically interact, while, at the same time, *OBSL1* and *CUL7* interact; however, in this system *CCDC8* and *CUL7* do not appear to directly associate [27]. The identification of three different genes associated with the same growth disorder and the associated physical interactions between the three proteins would suggest that they exist within a common biochemical pathway.

How Does Disruption of the *CUL7/OBSL1* Pathway Lead to Growth Restriction?

Murine models of *Cul7* and *Fbxw8* disruption exhibit an impairment of growth, and cell lines derived from these animal models demonstrate a reduction in cell proliferation and induction of senescence [31, 33, 36]. It is likely that the primary mechanism responsible for the growth restriction seen in knockout mouse models and in 3-M syndrome patients is the disruption of the *CUL7* SCF complex. The downstream effects of this disruption result in a failure of IRS-1 ubiquitination and proteolysis, thus leading to cellular accumulation of IRS-1.

Alteration of the IGF-I pathway has long been shown to result in severe growth restriction in mouse knockout

models [52–54]. The apparent accumulation of IRS-1 leads to the activation of the AKT and MAPK pathways. It is postulated that the overstimulation of these pathways ultimately leads to induction of cellular senescence [36], which in turn could result in poor growth explaining the growth restriction of 3-M syndrome patients.

Future studies should be directed at the identification of the remaining members of this pathway and how this novel pathway controls cell growth. For instance, it is currently unclear as to how *OBSL1* interacts with the IGF-I signalling pathway; however, it may function as a structural protein linking *CUL7* to IRS-1. The identification of further genes causing 3-M syndrome is likely to reveal other proteins in this pathway.

The importance of this pathway extends beyond human growth in that *CUL7* has been implicated in cancer. It has been shown in a number of studies that the expression of SCF complexes, notably *CUL1* (reviewed in [55]) and *CUL7* [44], are elevated in a number of malignant tumours, but it may be that loss of *CUL7* and/or associated proteins is in fact cancer protecting. Thus, understanding the key interactions between *CUL7*, *OBSL1*, *CCDC8* and IGF signalling may have implications both for statural growth and dysregulated cellular growth.

Acknowledgments

Support from the National Institute of Health Research Manchester Biomedical Research Centre is acknowledged. P.G.M. is a Medical Research Council (UK) clinical research fellow.

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