USP7 Acts as a Molecular Rheostat to Promote WASH-Dependent Endosomal Protein Recycling and Is Mutated in a Human Neurodevelopmental Disorder

Highlights

- USP7 is part of the MAGE-L2-TRIM27 ubiquitin ligase and enables endosomal recycling
- USP7 protects TRIM27 from auto-ubiquitination and proteasomal degradation
- USP7 buffers WASH ubiquitination levels to maintain proper endosomal actin levels
- Mutation of USP7 causes a human neurodevelopmental syndrome, including autism

In Brief

Hao et al. describe a function of the USP7 deubiquitinating enzyme in regulation of WASH/retromer-mediated endosomal protein recycling. USP7 functions as a molecular rheostat to prevent auto-ubiquitination and proteasomal degradation of TRIM27 E3 ubiquitin ligase, but also deubiquitinates WASH. Genetic studies identify cases of USP7 mutation/deletion resulting in a human neurodevelopmental disorder that overlaps with MAGE-L2 mutation.

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USP7 Acts as a Molecular Rheostat to Promote WASH-Dependent Endosomal Protein Recycling and Is Mutated in a Human Neurodevelopmental Disorder

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SUMMARY

Endosomal protein recycling is a fundamental cellular process important for cellular homeostasis, signaling, and fate determination that is implicated in several diseases. WASH is an actin-nucleating protein essential for this process, and its activity is controlled through K63-linked ubiquitination by the MAGE-L2-TRIM27 ubiquitin ligase. Here, we show that the USP7 deubiquitinating enzyme is an integral component of the MAGE-L2-TRIM27 ligase and is essential for WASH-mediated endosomal actin assembly and protein recycling. Mechanistically, USP7 acts as a molecular rheostat to precisely fine-tune endosomal F-actin levels by counteracting TRIM27 auto-ubiquitination/degradation and preventing overactivation of WASH through directly deubiquitinating it. Importantly, we identify de novo heterozygous loss-of-function mutations of USP7 in individuals with a neurodevelopmental disorder, featuring intellectual disability and autism spectrum disorder. These results provide unanticipated insights into endosomal trafficking, illuminate the cooperativity between an ubiquitin ligase and a deubiquitinating enzyme, and establish a role for USP7 in human neurodevelopmental disease.

INTRODUCTION

Post-translational modification of proteins by ubiquitin serves as an important cellular regulatory event to spatially and temporally control the stability and activity of proteins (Komander and Rape, 2012). Ubiquitination is highly regulated and controlled by E3 ubiquitin ligases (Deshaies and Joazeiro, 2009). However, equally important is the counterbalance by deubiquitinating enzymes that reverse the action of ubiquitin ligases (Komander et al., 2009; Reyes-Turcu et al., 2009). How these two seemingly opposing activities work in concert to allow precise control of cellular signaling and biological processes is not fully resolved. Ubiquitin-specific protease 7 (USP7; also known as herpesvirus-associated ubiquitin-specific protease, HAUSP) is a deubiquitinating enzyme in the ubiquitin-specific protease family that can cleave multiple chain linkages, including short K48-linked ubiquitin chains and longer K63-linked ubiquitin chains (Li et al., 2002; Nicholson and Suresh Kumar, 2011). USP7 regulates the ubiquitination of many proteins, including the MDM2-p53 pathway, that impacts a number of diverse cellular and pathophysiological processes, such as DNA repair, transcription, immune responses, viral replication, and cancer (Li et al., 2002; Nicholson and Suresh Kumar, 2011; Schaefer and Morgan, 2011). USP7 regulates the ubiquitination of many proteins, including the MDM2-p53 pathway, that impacts a number of diverse cellular and pathophysiological processes, such as DNA repair, transcription, immune responses, viral replication, and cancer (Li et al., 2002; Nicholson and Suresh Kumar, 2011). Knockout of Usp7 in mice results in early embryonic lethality that cannot be rescued by p53 knockout, suggesting additional roles of Usp7 (Kon et al., 2010).

MAGE-L2 is part of the melanoma antigen gene (MAGE) family of E3 ubiquitin ligase regulators (Doyle et al., 2010). MAGE-L2
bonds to the TRIM27 ubiquitin ligase and mediates its recruitment to endosomes where it facilitates retromer-dependent recycling of proteins from endosomes back to the trans-Golgi network (retrograde; such as CI-M6PR) or to the plasma membrane (such as integrins) (Burd and Cullen, 2014; Hao et al., 2013). The MAGE-L2-TRIM27 ubiquitin ligase promotes endosomal protein recycling by K63-linked poly-ubiquitination of WASH, an actin nucleation promoting factor essential for this fundamental recycling pathway (Derivery et al., 2009; Gomez and Billadeau, 2009; Hao et al., 2013; Seaman et al., 2013). Non-degradative ubiquitination of WASH leads to its activation through unlocking an auto-inhibited state, resulting in endosomal actin assembly and protein recycling (Hao et al., 2013; Jia et al., 2010). Importantly, MAGEL2 is one of the protein-coding genes typically deleted in Prader-Willi syndrome (PWS), a human neurodevelopmental disease (Boccaccio et al., 1999; Lee et al., 2000). Furthermore, truncating mutations of MAGEL2 result in Schaaf-Yang syndrome (MIM 615547) with marked clinical overlap to PWS, highlighted by hypotonia, hypogonadism, intellectual disability, and autism spectrum disorder (Schaaf et al., 2013). The Magel2 knockout mice recapitulate several of these phenotypes (Bischof et al., 2007; Kozlov et al., 2007; Mercer et al., 2009; Mercer and Wevrick, 2009; Tennese and Wevrick, 2011). This suggests that the regulation of WASH-mediated endosomal actin assembly and protein recycling by the MAGE-L2-TRIM27 ubiquitin ligase plays an important role in neurodevelopment.

RESULTS

USP7 Is an Integral Component of the MAGE-L2-TRIM27 Ubiquitin Ligase Complex

To identify MAGE-L2-TRIM27 regulatory factors, we performed tandem affinity purification of MAGE-L2 followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). As expected, MAGE-L2 co-purified with the E3 ubiquitin ligase TRIM27 (Figure 1A). Strikingly, we unexpectedly found USP7 co-purifying with MAGE-L2 (Figure 1A). The robust interaction of USP7 with MAGE-L2 (and TRIM27) was confirmed by co-expression and endogenous immunoprecipitation experiments (Figures 1B and 1C). Expression of GFP-tagged USP7 in U2OS cells revealed co-localization with mCherry-TRIM27 cytoplasmic puncta and the WASH complex (FAM21), although the majority of GFP-USP7 localizes to the nucleus (Figure 1D). In vitro experiments using recombinant proteins revealed that USP7 not only directly bound MAGE-L2, but also directly bound TRIM27 (Figure 1E), consistent with previous results (Zaman et al., 2013). Through a series of in vitro binding studies and cellular co-immunoprecipitation experiments, we find that MAGE-L2 interacts with USP7 through two distinct interfaces, most prominently with the USP7 N-terminal TRAF domain and more weakly with the C-terminal HUBL1-3 regulatory domains (Faesen et al., 2011) (Figures S1A–S1D and summarized in Figure 1F). In contrast, TRIM27’s C-terminal domains interact with the catalytic domain of USP7 (Figures S1A–S1D and summarized in Figure 1F). Consistent with the importance of USP7 in proper assembly of the complex, attempts to purify the MAGE-L2-TRIM27 complex failed in the absence of co-expression and purification with USP7 in insect cells (Figure S1E). However, co-expression of all three subunits of the complex yielded stoichiometric amounts of MAGE-L2, TRIM27, and USP7 (Figure S1F). These results suggest that MAGE-L2, TRIM27, and USP7 form an intricate and stable protein complex.

USP7 Is Required for WASH-Mediated Endosomal Actin Accumulation and Protein Recycling

Given that MAGE-L2-TRIM27 promotes WASH activation, endosomal actin assembly, and protein recycling through WASH ubiquitination (Hao et al., 2013), we hypothesized that USP7 may inhibit these processes by counteracting MAGE-L2-TRIM27’s activity. Surprisingly, knockdown of USP7 had the opposite effect, resulting in phenotypes that mimicked MAGE-L2, TRIM27, or WASH knockdown, including impairment of CI-M6PR protein recycling (Figures 2A, 2B, and S2A) that resulted in endosomal accumulation of CI-M6PR (Figure S2B). Importantly, these phenotypes were evident in both p53-proficient (U2OS, Figures 2A and 2B) and deficient (HeLa, Figures S2C and S2D) cells and were not due to defects in the general trans-Golgi network architecture (Figure S2E). Additionally, knockdown of USP7 impaired retrograde trafficking of other WASH/retromer-dependent proteins, including cell surface CI-M6PR (Figure 2C), cholera toxin subunit B (CtxB; Figures 2D, 2E, and S2F), and TGN46 (Figure S2G). Depletion of USP7 also impacted WASH-dependent recycling of Integrin α5 to the plasma membrane (Figure S2H). However, other WASH-independent endosomal recycling pathways, such as trafficking of transferrin, were unaffected by USP7 knockdown (Figures 2D and S2F). Consistent with the impaired trafficking of WASH/retromer-dependent proteins due to alterations in WASH activity, knockdown of USP7 decreased levels of F-actin and the Arp2/3 subunit ArpC5 (Figures 2F–2I) on FAM21/VPS35-positive endosomes to a degree similar to knockdown of MAGE-L2 or WASH. Knockdown of USP7 had no general effect on total cellular levels of ArpC5 (Figure 3A). Importantly, defects in CI-M6PR retrograde transport in USP7-RNAi cells could be rescued by elevating endosomal F-actin levels using an endosomal-targeted active WASH construct we previously characterized (Figure 2J) (Hao et al., 2013).

USP7 is known to deubiquitinate and regulate several proteins. To determine whether its role in endosomal actin assembly and WASH-dependent protein trafficking was through its interaction with MAGE-L2-TRIM27, we identified a MAGE-L2 mutant that disrupted USP7 interaction. MAGE-L2 (amino acids 820–1034) interacts with the USP7 TRAF domain (Figures 1F and S1), which has previously been shown to bind to a conserved motif A/P-X-X-S (Sheng et al., 2006). Within this portion of MAGE-L2, six TRAF binding motifs were identified (Figure S2I). Ser to Ala mutation of these motifs (MAGE-L2 SA) blocked USP7 interaction (Figure 2K). MAGE-L2 SA mutant also showed decreased binding to TRIM27, suggesting that USP7 facilitates MAGE-L2-TRIM27 complex formation in cells (Figure 2K). Importantly, knockdown of endogenous MAGE-L2 and expression of MAGE-L2 SA disrupted CI-M6PR endosomal protein recycling (Figures 2L and S2J), Arp2/3 localization to FAM21-positive endosomes (Figure 2M), and F-actin accumulation on FAM21-positive endosomes (Figure 2N). Taken together, these findings...
suggest that the USP7 deubiquitinating enzyme forms a stable complex with the MAGE-L2-TRIM27 E3 ubiquitin ligase and that all three proteins work in concert to control WASH-mediated actin assembly and protein recycling.

**USP7 Prevents Auto-ubiquitination and Degradation of TRIM27**

To investigate how USP7 supports MAGE-L2-TRIM27 and WASH-mediated protein recycling, we investigated whether USP7 controls the steady-state levels or localization of any of the known components of the endosomal protein recycling pathway, including the important WASH actin assembly and retromer coat-like complexes. WASH and retromer complexes localized properly on endosomes (Figure S3) and their protein levels were unaffected by USP7 knockdown (Figure 3A). However, TRIM27 protein levels were dramatically decreased (Figure 3A). This result was confirmed to be an on-target effect using multiple siRNAs targeting USP7 and knockout of USP7 in HCT116 cells (USP7<sup>−/−</sup>) (Cummins et al., 2004) (Figures 3A and 3B). Importantly, these effects were independent of p53 status and could be rescued by the MG132 proteasome inhibitor (Figures 3C and 3D), suggesting that USP7 prevents ubiquitin-mediated degradation of TRIM27 by the proteasome. To determine whether USP7 stabilizes TRIM27 through its deubiquitinating activity, we suppressed endogenous USP7 and re-expressed wild-type or a USP7 mutant (C233S) that lacks...
Figure 2. USP7 Is Required for WASH-Mediated Endosomal Protein Recycling

(A and B) Knockdown of USP7 disrupts CI-M6PR recycling. U2OS cells were treated with the indicated siRNAs for 72 hr followed by immunostaining (A) and quantification (B).

(C) Trafficking of cell-surface-labeled CI-M6PR is impaired upon knockdown of USP7. U2OS cells were treated with indicated siRNAs for 72 hr before cell surface CI-M6PR was labeled with anti-CI-M6PR for 30 min. Cells were fixed at the indicated time points after labeling, the internalized CI-M6PR was detected, and the percentage of cells with juxtanuclear internalized CI-M6PR was determined.

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USP7 Acts as a Molecular Rheostat to Control WASH Activity and Endosomal Actin Assembly through Deubiquitination of WASH

Next, we examined whether USP7’s sole role in endosomal protein recycling was stabilization of TRIM27. To do so, we identified a non-deubiquitinatable mutant of TRIM27 that was stable even in the absence of USP7. First, we mutated all lysines in TRIM27 to arginine (termed, all K-R) and examined whether this TRIM27 mutant was resistant to USP7 knockdown. This was indeed the case (Figures 4A and S4A). To further refine which lysines in TRIM27 contribute to its auto-deubiquitination and instability in the absence of USP7, we mapped its ubiquitination sites by anti-diGly affinity proteomics which revealed five ubiquitination sites (K79, K292, K304, K380, and K382) (data not shown). Mutation of these individually or in various combinations ultimately identified a TRIM27 3KR mutant (K304R, K380R, and K382R) that was stable even in the absence of USP7 (Figures 4A and S4A and data not shown). Importantly, TRIM27 3KR incorporated into the MAGE-L2-TRIM27-USP7 complex (Figures 4B and S4B) and is fully functional in supporting CI-M6PR recycling (Figure 4C). Thus, TRIM27 3KR allowed us to uncouple USP7’s activity on stabilizing TRIM27 from any other potentially unknown activity of USP7 on this pathway. We determined whether cells reconstituted with TRIM27 3KR mutant are resistant to USP7-RNAi-induced defects in CI-M6PR recycling, as would be expected if USP7’s only role was stabilization of TRIM27. However, this was not the case as knockdown of USP7 in TRIM27 3KR cells still impaired CI-M6PR recycling similarly to TRIM27 wild-type cells (Figure 4D). Analysis of these cells revealed that knockdown of USP7 in TRIM27 3KR cells significantly increased Arp2/3 and F-actin levels on FAM21-positive endosomes (Figures 4E and 4F). This lead us to hypothesize that in addition to preventing TRIM27 auto-ubiquitination and degradation, USP7 also prevents over-activation of WASH and improper CI-M6PR recycling by precisely tuning WASH ubiquitination and endosomal actin levels. Consistent with this hypothesis, the level of ubiquitinated WASH is increased upon knockdown of USP7 in TRIM27 3KR cells (Figure 4G). Furthermore, ubiquitination of WASH in vitro by MAGE-L2-TRIM27 was restrained by wild-type USP7 when compared to inactive USP7 C233S mutant (Figure 4H). Taken together, these results suggest that USP7 limits overproduction of endosomal F-actin by WASH through its ability to deubiquitinate WASH (Figure 4I) and that precise endosomal F-actin levels are critical for proper endosomal protein recycling. Consistent with this, artificial elevation of endosomal F-actin (Figures 4J and S4C) by overexpression of the WASH K220D mutant that mimics the activation of WASH by ubiquitination (Hao et al., 2013) causes defects in CI-M6PR recycling (Figures 4K and S4D).

Disruption of USP7 in Humans Is Associated with a Neurodevelopmental Disorder, Featuring Intellectual Disability and Autism Spectrum Disorder

Given our identification of USP7 as a critical component of the MAGE-L2-TRIM27 ligase complex that regulates WASH-mediated endosomal protein recycling and our previous identification of truncating MAGEL2 mutations in children with Schaaf-Yang syndrome (Schaaf et al., 2013), we hypothesized that mutations in USP7 may lead to similar clinical phenotypes. We queried clinical databases of chromosome microarray analyses on approximately 94,000 individuals (Baylor College of Medicine Molecular Genetics Laboratory and Signature Genomic Laboratories, February 2014), the publically available DECIPHER (Database of genomic variation and Phenotype in Humans using Ensembl Resources) database, and the database of clinical whole exome sequencing at the Whole Genome Laboratory of Baylor College of Medicine (approximately 1,500 cases at the time of query, i.e., February 2014). We identified six cases with heterozygous chromosomal microdeletions (see Figure 5A and Table S1 for

(D and E) Depletion of USP7 impairs cholera toxin subunit B (CTxB), but not transferrin (Tf) trafficking. U2OS cells were treated with the indicated siRNAs for 72 hr before fluorescently labeled CTxB and Tf were added to cells for 15 min. Cells were fixed at the indicated time points post-labeling and the percentage of cells with CTxB and Tf localized with p97-golgin was determined by counting (D) or Pearson correlation (E).
(F–I) Knockdown of USP7 decreases endosomal F-actin and Arp2/3 levels. U2OS cells were treated with the indicated siRNAs for 72 hr followed by staining for F-actin (Phalloidin) and endosomal marker FAM21 (F) and quantification (G) or ArpC5 and endosomal marker VPS35 (H) and quantification (I).
(J) USP7-RNAi induced defects in CI-M6PR recycling are rescued by expression of the WASH K220D mutant that mimics the activation of WASH by ubiquitination (Hao et al., 2013) causes defects in CI-M6PR recycling (Figures 4K and S4D).
and one case with a heterozygous nonsense mutation in USP7 (Figure 5A and Table 1). Subsequent testing revealed that all seven mutations were de novo (p < 0.0001, two-tailed Chi-square analysis with Yates’ correction and Bonferroni correction for multiple comparisons, see supplemental experimental procedures). In a genotype-to-phenotype approach, we obtained detailed clinical information on the affected individuals (Table 1 and Supplemental Clinical Notes),

**Figure 3. USP7 Protects TRIM27 from Auto-ubiquitination-Induced Degradation**

(A) Knockdown of USP7 dramatically reduces TRIM27 levels without affecting WASH and retromer complex levels. HeLa cells were treated with the indicated siRNAs for 72 hr before immunoblotting for the indicted proteins. Asterisks indicate non-specific bands.

(B) USP7 knockout (USP7+/C0/C0) cells have reduced TRIM27 protein levels. Cell lysates from the indicated HCT116 cells were immunoblotted.

(C and D) Proteasome inhibitor (MG132) rescues TRIM27 protein levels upon USP7 knockdown. p53-proficient U2OS (C) and p53-deficient HeLa (D) cells were treated with the indicated siRNAs for 72 hr, incubated with DMSO (vehicle) or MG132 for 4 hr, and immunoblotted.

(E) USP7 deubiquitinating enzymatic activity is critical for maintenance of TRIM27 protein levels. HeLa cells were treated with the indicated siRNAs for 24 hr before expression of the indicated constructs for 48 hr followed by immunoblotting for the indicated proteins.

(F) USP7 deubiquitinates TRIM27 auto-ubiquitination in vitro. Purified proteins were used in in vitro ubiquitination reactions before samples were separated by SDS-PAGE and anti-TRIM27 immunoblotting was performed.

Results are representative of at least three replicate experiments. See also Figure S3.
which revealed striking overlap with the phenotypes observed in individuals with MAGEL2 loss-of-function mutations. In summary, the most prevalent phenotypes were intellectual disability (100%), autism spectrum disorder (83%), epilepsy (71%), aggressive behavior (57%), hypotonia (57%), and hypogonadism (80%). These results suggest haploinsufficiency of USP7 as a mechanism for pathogenesis in human neurodevelopment.

**USP7 Is Haploinsufficient for Its Specific Function in Endosomal Protein Recycling**

Given these findings, we next investigated whether USP7 is haploinsufficient for its role in regulating TRIM27 protein levels and WASH-mediated endosomal protein recycling on a molecular basis. Partial knockdown of USP7 resulted in a significant decrease in TRIM27 protein levels and impairment of CI-M6PR recycling (Figures 5B and SSA). Furthermore, knockout of one USP7 allele in HCT116 cells (USP7+/−) resulted in impaired CI-M6PR recycling (Figures 5C and 5D), decreased Arp2/3 localization to VPS35-positive endosomes (Figures 5E and 5S), and decreased F-actin accumulation on VPS35-positive endosomes (Figures 5F and 5C). Importantly and as reported previously (Cummins and Vogelstein, 2004; Kon et al., 2010), many of the other functions of USP7, including regulation of the p53 pathway, were not dramatically altered in USP7+/− cells as compared to USP7−/− cells (Figure 5G). Furthermore, specific abrogation of the p53 regulatory pathway by knockdown of HDM2 or HDMX did not impair localization of Arp2/3 or accumulation of F-actin on FAM21-positive endosomes (Figures S6D–SS). Consistent with these findings, knockdown of MAGE-L2 or TRIM27 did not impact other functions of USP7, such as regulation of the MDM2-p53 pathway (Figure S6G). Additionally, reconstitution of USP7−/− cells with USP7 Y143X found in subject #6 failed to rescue CI-M6PR recycling or rescue Arp2/3 and F-actin levels on FAM21-positive endosomes (Figures 5H–5J). These results, in combination with the similarities to clinical manifestations of children with MAGEL2 mutation, suggest that disruption of the MAGE-L2-TRIM27-USP7 WASH regulatory pathway is associated with Schaaf-Yang syndrome phenotypes in humans, prominently featuring intellectual disability and autism spectrum disorder.

**MAGE-L2-TRIM27-USP7 Plays an Important Role in Hypothalamic Neurons**

To begin to understand how disruption of the MAGE-L2-TRIM27-USP7 regulatory axis controlling retromer and WASH-mediated endosomal protein recycling may contribute to disease, we investigated which anatomical tissues may be critically dependent on this regulatory pathway by examining if MAGE-L2, TRIM27, or USP7 are enriched in specific tissues. Unlike the ubiquitous TRIM27 and USP7 genes (Figures S6A and S6B) that have functions independent of regulation of WASH, MAGE-L2 is highly enriched in the brain of both mice and humans (Figures 6A–6C). Detailed analysis of the various regions of the mouse brain revealed that MAGE-L2 is highly enriched in the hypothalamus (Figures 6A and 6B). Intriguingly, the hypothalamus is strongly implicated in several of the clinical symptoms associated with MAGE-L2 and USP7 deletion/mutation, including PWS and autism (Cassidy et al., 2012; Jacobson, 2014; Tennese and W ervick, 2011; Wetsel et al., 1991). Therefore, we examined whether MAGE-L2-TRIM27-USP7 plays an important role in regulation of WASH in hypothalamic neurons that endogenously express MAGE-L2 (Miller et al., 2009). Partial knockdown of USP7 resulted in reduction in TRIM27 protein levels (Figure 6D) and impairment in Arp2/3 localization (Figures 6E and 6F) and F-actin assembly (Figures 6E and 6G) on FAM21-positive endosomes. Knockdown of other genes within the deletion regions around the USP7 genomic locus (Figure 5A) did not decrease TRIM27 protein levels (Figure S6C) or levels of Arp2/3 on FAM21-positive endosomes (Figure S6D). Furthermore, CRISPR/Cas9 knockout of USP7 in hypothalamic neurons resulted in similar phenotypes, including reduced TRIM27 protein levels (Figure 6H) and diminished the level of F-actin on...
A. Chromosome 16

B. Dispersed CI-M6PR (% of Cells)

C. CI-M6PR + DNA

D. Dispersed CI-M6PR (% of Cells)

E. ArpC5 Intensity on VPS35 Endosomes (RFU)

F. F-Actin Intensity on VPS35 Endosomes (RFU)

G. +/+ +/+ Usp7

H. Dispersed CI-M6PR (% of Cells)

I. ArpC5 Intensity on FAM21 Endosomes (RFU)

J. F-Actin Intensity on FAM21 Endosomes (RFU)

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FAM21-positive endosomes (Figures 6I and 6J). Therefore, MAGE-L2-TRIM27-USP7 may be critically important for proper hypothalamic function, and disruption of this pathway may contribute to the development of Schaaf-Yang syndrome with its various phenotypic manifestations.

**DISCUSSION**

Ubiquitination plays many important regulatory roles during the complex process of endosomal protein transport, including endocytosis, sorting and targeting of proteins to multi-vesicular bodies, and regulation of recycling pathways. Previous studies have implicated multiple deubiquitinating enzymes in these processes, including USP8 that is required for retrograde transport of CI-M6PR (MacDonald et al., 2014). We provide evidence that USP7 functions in the endosomal protein recycling pathway through its incorporation into the MAGE-L2-TRIM27 ubiquitin ligase complex. Intriguingly, USP7 has dual activities: (1) promoting WASH ubiquitination by preventing TRIM27 auto-ubiquitination and degradation through its deubiquitinating activity and (2) limiting WASH ubiquitination through direct deubiquitination of WASH. These seemingly opposing activities of USP7 allow for precision control and tuning of WASH activity. This is critically important because endosomal F-actin follows the "Goldilocks principle" in which too little or too much is detrimental for retro-mer-dependent protein recycling (Gomez and Billadeau, 2009; Hao et al., 2013) (Figure 4). In this way, USP7 acts to buffer WASH ubiquitination to maintain optimal activity and endosomal F-actin levels. This level of cooperativity observed between USP7 and MAGE-L2-TRIM27 may extend to other DUB-E3 ligase pairs.

In addition to defining a role for USP7 in cellular protein trafficking and its mechanisms of action, we report a genetic

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**Table 1. Mutation Summary and Clinical Phenotypes of Seven Individuals with USP7 Mutations**

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<td>+</td>
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<td>+</td>
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<td>5/7</td>
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<tr>
<td>Cryptorchidism/micropenis</td>
<td>+</td>
<td>+</td>
<td>N/A</td>
<td>–</td>
<td>+</td>
<td>N/A</td>
<td>+</td>
<td>4/5</td>
</tr>
<tr>
<td>Hypotonia</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
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<td>+</td>
<td>–</td>
<td>4/7</td>
</tr>
<tr>
<td>Aggressive behavior</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>4/7</td>
</tr>
</tbody>
</table>

+, present; –, not present; b, base; Del, deletion; DD/ID, developmental delay/intellectual disability; F, female; kb, kilobases; M, male; Mb, megabases; N/A, not applicable; o, unknown. See also Table S1.

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FAM21-positive endosomes (Figures 6I and 6J). Therefore, MAGE-L2-TRIM27-USP7 may be critically important for proper hypothalamic function, and disruption of this pathway may contribute to the development of Schaaf-Yang syndrome with its various phenotypic manifestations.
disorder due to disruption of USP7. We show that heterozygous deletion or mutation of USP7 results in a number of neurological and behavioral phenotypes. Importantly, there is marked clinical overlap with Schaaf-Yang syndrome caused by MAGE-L2 loss-of-function, such as hypotonia, developmental delay/intellectual disability, autism spectrum disorder, and hypogonadism. Other phenotypes appear to be more distinct to USP7 haploinsufficiency, i.e., seizures and aggressive behaviors. Conversely, some phenotypes characteristic of PWS were not associated with deletion or mutation of USP7, namely infantile feeding difficulties, excessive weight gain, hyperphagia, and distinct craniofacial features. This suggests a spectrum of genotype-phenotype correlations with classic PWS on one side, MAGEL2-associated Schaaf-Yang syndrome, and now USP7-associated disease at the other end of the spectrum. Consistent with our findings, a genome-wide association study implicated TRIM27 in autism spectrum disorder (St Pourcain et al., 2013). Additionally, genomic duplications involving USP7 were reported in individuals with autism spectrum disorder (Sanders et al., 2011), further supporting the idea of dosage sensitivity, with both too little and too much USP7 causing imbalances of neuronal homeostasis. Furthermore, conditional knockout of USP7 in the mouse brain results in neonatal lethality that cannot be fully rescued by p53 knockout (Kon et al., 2011). This is consistent with our failure to identify individuals homozygous null for USP7 and our findings suggesting that USP7 has critical p53-independent functions in the brain.

Our results indicate that the disruption of USP7-mediated regulation of MAGE-L2-TRIM27 and WASH impairs endosomal protein recycling and F-actin assembly. This cellular function of USP7 may contribute to the described USP7-associated disease, although we cannot exclude that some of the phenotypes may be attributed to other nuclear functions of USP7 or TRIM27, such as regulation of TNFα-induced cell death through deubiquitination of RIP1 (Zaman et al., 2013). Similar to the linkage of endocytic trafficking to neurodegenerative disorders, including mutation of the retromer subunit VPS35 in Parkinson disease (Vilarin˜o-Gu¨ell et al., 2011), our findings imply that alterations in recycling of membrane proteins in the brain may contribute to impaired neurological and cognitive functions. Consistently, a role for endosomal trafficking proteins in a neurodevelopmental disorder of the mouse was recently reported (Watson et al., 2015). Finally, our results suggest that chemically activating WASH in these patients may have therapeutic potential.

EXPERIMENTAL PROCEDURES

Cell Culture, Transfections, CRISPR/Cas9, and Antibodies

GT1-7 hypothalamic neuronal cells (kindly provided by Pamela Mellon, UCSD) (Wetsel et al., 1991), HCT116 USP7+/+, USP7+/−, USP7−/− cells (kindly provided by Bert Vogelstein, Johns Hopkins University) (Cummins et al., 2004), HEK293 (ATCC), HeLa Tet-ON (Clontech), or U2OS (ATCC) cells were grown in DMEM (Invitrogen) supplemented with 10% FBS (HyClone), 2 mM L-Glutamine (Invitrogen), 100 units/ml penicillin (Invitrogen), 100 mg/ml streptomycin (Invitrogen), and 0.25 mg/ml amphotericin B (Invitrogen). Plasmid and siRNA transfection were performed for 48–96 hr with Effectene (QIAGEN) or Lipofectamine RNAiMAX (Invitrogen), respectively, according to the manufacturer’s protocol. siRNAs (GE Dharmacon or Sigma) and antibodies used in this study are listed in the Supplemental Experimental Procedures. USP7 knockout GT1-7 cells were generated by co-transfection of plasmids encoding GFP-Cas9 and a gRNA plasmid targeting USP7 (5’-GGTTGGCTCGAGACGCCC-3’). Forty-eight hours after transfection, cells were flow sorted for GFP-positive cells. USP7 disruption was confirmed by western blotting.

Tandem Affinity Purification, Immunoprecipitation, and Immunoblotting

Tandem affinity purification (TAP) was performed using 293/TAP-Vector or 293/TAP-MAGE-L2 stable cell lines as described previously (Doyle et al., 2010) and detailed in the Supplemental Experimental Procedures. Immunoprecipitation and immunoblotting were performed as described previously (Potts and Yu, 2005) and in the Supplemental Experimental Procedures.

In Vitro Binding Assays

Proteins were purified from bacterial or SF9 cells using standard protocols and described in detail in the Supplemental Experimental Procedures. In vitro binding assays were performed as described previously (Doyle et al., 2010), with details in the Supplemental Experimental Procedures.

Immunofluorescence, Microscopy, and Quantitative Measurements

Immunofluorescence was performed essentially as described previously (Hao et al., 2013). Cells were imaged with a 63× or 100× objective on a DeltaVision or Nikon TiU inverted fluorescence microscope. Images were acquired with a CoolSnap HQ2 charge-coupled device camera (Photometrics) at 0.3-μm intervals, deconvolved using the nearest neighbor algorithm, and stacked to better resolve endosome structures. Intensity measurements were performed using ImageJ Software. For those experiments specifically quantitating the amount of endosomal localized F-Actin or Arp2/3, images (> 100 cells over multiple independent experiments) were thresholded in ImageJ to create a mask (or region of interest) for the relevant endosomes (anti-FAM21 or anti-VPS35 signal) and the relative fluorescent units intensity of F-Actin (phalloidin) or anti-Arp2/3 signal overlapping with FAM21 or VPS35 in the thresholded (masked) region was determined. The

Figure 6. MAGE-L2-TRIM27-USP7 Plays Critical Role in the Hypothalamus

(A and B) RT-QPCR analysis reveals that MAGE-L2 is highly enriched in the hypothalamus of both C57BL/6 (A) and BALB/c (B) mice. The indicated tissues were collected from six mice, pooled, and RT-QPCR analysis of MAGE-L2 expression was performed and normalized to 18S rRNA.

(C) Expression of human MAGE-L2 is enriched in the brain. RNA from the indicated tissues was obtained and RT-QPCR analysis of MAGE-L2 expression was performed and normalized to 18S rRNA.

(D) Knockdown of USP7 in hypothalamic neurons significantly decreases TRIM27 protein levels. Gt1-7 hypothalamic neurons were treated with the indicated siRNAs for 72 hr before cell lysates were immunoblotted for the indicated proteins.

(E–G) Knockdown of MAGE-L2 or USP7 in hypothalamic neurons impairs F-actin accumulation (E and F) and Arp2/3 endosomal localization (E and G). Gt1-7 hypothalamic neurons were treated with the indicated siRNAs for 72 hr before immunostaining (E and G).

(H–J) CRISPR/Cas9 knockout of USP7 decreases TRIM27 protein levels (H) and levels of F-actin on FAM21-positive endosomes (I–J) levels. Gt1-7 hypothalamic neurons were transfected with GFP-Cas9 and the indicated gRNAs for 48 hr before GFP-positive cells were collected by flow cytometry and analyzed by immunoblotting (H) and immunostaining (I and J) after culturing.

Results are representative of at least three replicate experiments. More than 100 cells were quantitated. Values shown are mean ± SD. Asterisks indicate p < 0.05. Scale bar represents 20 μm. See also Figure S6.
threshold for endosomal markers was kept constant across all images analyzed to prevent bias. Quantitation of CI-M6PR trafficking was performed by blind analysis of the percentage of cells showing compact juxtanuclear (normal) or dispersed (abnormal) CI-M6PR staining. For colocalization analysis, Pearson correlation measurements were performed using ImageJ colocalization package. At least 100 cells were counted for each condition in each experiment. Statistical analysis was performed using two-tailed unpaired Student’s t test.

**In Vitro Ubiquitination Assay**

In vitro ubiquitination assays were performed as described previously (Doyle et al., 2010). Assays were performed using 100 nM His-Ube1 (Biomol), 2.5 μM E2 (His-UbCH5), 150 nM E3 (His-MAGE-L2/MBP-TRIM27/MBP- USP7 WT or C223S mutant), 2.5 μM ubiquitin (Biomol), 25 mM substrate (GST-WASH), 0.5 mM Mg-ATP (Biomol), 20 units/ml inorganic pyrophosphatase (Sigma), and 1 mM DTT in 1x ubiquitylation buffer (Biomol). Negative control reactions were set up as described above except Mg-ATP was replaced with 5 mM EDTA. Reactions were stopped by the addition of SDS sample buffer after incubation at 30°C for 2 hr and subjected to SDS-PAGE and immunoblotting for auto-ubiquitination of TRIM27 and ubiquitination of WASH.

**Human Subjects**

Clinical chromosome microarray databases (Baylor College of Medicine Molecular Genetics Laboratory, BCM-MGL, and Signature Genomic Laboratories), the publically available DECIPHER (Database of genomiC varia
tion and Phenotype in Humans using Ensembl Resources) database, and the database of clinical whole exome sequencing at the Whole Genome Laboratory (WGL) of Baylor College of Medicine were queried in February 2014. The Baylor and Signature Genomic chromosome microarray data bases contained samples of 94,242 individuals combined at that time, while the DECIPHER database contained approximately 10,000 individuals at the time of query. We limited our search on those smaller than 5 Mb in size. Seven cases were identified, six of which were successfully enrolled in this study (Signature Genomics: subject 1; DECIPHER: subjects 2 and 7; BCM-MGL: subjects 3, 4, and 5). The WGL database of exome sequencing encompassed approximately 1,500 cases at the time of query. Importantly, these are consecutive, unrelated samples without prescreening criteria. Samples are submitted as a clinical test, on the referring physician’s determination that the patient likely has a genetic change that has led to genetic disease. As part of a study approved by the Institutional Review Board of Baylor College of Medicine, the referring physicians of subjects 1, 3, 4, 5, and 6 were re-contacted, and families granted permission to publish genomic and phenotypic information. For subjects 2 and 7, patient consent was obtained by the attending geneticist for public and collaborative group data sharing, giving access to genomic and phenotypic data and patient reports. Following informed consent, we performed a comprehensive chart review of medical records. As well, providers were asked to fill out a clinical questionnaire. See Supplemental Clinical Notes for details on subjects.

**RNA Preparation and cDNA Synthesis**

Specifics regarding tissue collection from animals are described in supplemental experimental procedures. RNA was extracted using RNAStat60 (TelTest) according to the manufacturer’s directions. Total RNA was pooled in equal quantities for each tissue (n = 6). Genomic DNA contamination was eliminated by DNase I (Roche) treatment in 4.5 mM MgCl₂. cDNA for qPCR assays was prepared from 4 μg DNA-treated RNA using High Capacity cDNA Reverse Transcription kit (Life Technologies). Gene expression levels were measured as described in the Supplemental Experimental Procedures.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, Supplemental Clinical Notes, six figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.molcel.2015.07.033.

**AUTHOR CONTRIBUTIONS**

The project was conceptualized by P.R.P. Investigation was performed by Y.H., K.F.T., and P.R.P. Formal analysis was done by P.R.P., Y.H., K.F.T., C.P.S., and M.D.F. Resources were provided by F.X., W.B., S.L.K., A.P., J.A.R., C.L.C., B.I., I.D.K., S.E.N., J.P.P., T.M.M., R.M., R.C.P., and M.S.S. Data curation was performed by M.D.F. and C.P.S. The original manuscript was written by P.R.P. and Y.H. The manuscript was reviewed and edited by P.R.P., C.P.S., Y.H., K.F.T., M.D.F., J.A.R., and B.I. Data presentation/visualization was constructed by P.R.P., C.P.S., Y.H., K.F.T., and M.D.F. The project was supervised and administrated by P.R.P. and C.P.S. Funding for the project was acquired by P.R.P. and C.P.S.

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