スプライシングの標的配列に対し，アンチセンス核酸をpre-mRNAレベルで結合させ，エクソントラップを阻止し，正常なスプライシングに戻す，「アンチセンス療法」が有効ではないかと考えた（図2-B）。そこで，これらの標的配列に対し有効なアンチセンス核酸を網羅的に設計し，さまざまな細胞系に投与しスプライシングの是正を検討し，アンチセンス核酸のカクテル，AED（以降AEDカクテル）を選び出した。次に我々はビボモルフォリノ（Octa-Guanidine Morpholino：VMO）というアンチセンス核酸を用い，AEDカクテルをモデル動物及び患者細胞に投与し治療効果を検討した。患者筋芽細胞に対し，AEDカクテルを投与したところ，非投与マウスに比較し，糖鎖の回復を示唆する糖化型α DGの分子量の劇的な増加がみられた（図3-A）[2]。また尾静脈経由のモデルマウスへのAEDカクテル全身投与においても，Oマンノース型糖鎖の劇的な回復がみられた（図3-B）[2]。最後に，患者由来筋芽細胞を使いAEDカクテル投与によるラミニン凝集アッセイを行った。患者筋芽細胞では筋管でのα DGの発現は激減している。しかしAEDカクテル投与により，患者由来の筋管はα DGの糖鎖が正常レベルに回復し，正常と同程度の典型的なラミニンの凝集が観察された（図2-C）[2]。これらの結果はAEDカクテル投与により，筋管が機能的にも回復したことを示唆する。

おわりに

アンチセンス核酸を用いたスプライシング操作を標的とした治療法の一つに，もっとも頻度の高い筋ジストロフィーであるDuchenne型筋ジストロフィーをBecker型にするエクソンスキップ療法があげられる。この治療法は現在国際治験が進行しており最も実現可能な治療薬剤として注目されている[8]。今回われわれが開発した方法は，エクソントラップ阻害の原理に基づく治療法であり，エクソンスキップとは原理が異なり，FCMDの根本的分子標的治療に道を開くものである[2]。Duchenne型と異なり，患者のほとんどが同じ変異をもつため，FCMDに対するアンチセンス療法は，日本のすべてのFCMDの患者を対象に同一の方法で行えるものであり有望であると考える。今後医療応用の実現を目指したい。

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＜細胞ニュース＞

第39回日本股関節学会

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「Periacetabular Osteotomy Surgery using The Minimally Invasive Approach」
2. 松下　隆先生（帝京大学医学部整形外科学　主任教授）
「脆弱性骨折 現状と今後の対応」
3. Carlos Sancineto, M.D.（Hospital Italiano de Buenos Aires Orthopaedic Trauma Section Argentina）
The treatment of nonunion and malunion of acetabular fractures.
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「股関節バイオメカニクスに関する最近の話題」
5. Guoan Li, Ph.D.（Bioengineering Lab., Department of Orthopaedic Surgery, Massachusetts General Hospital and Harvard Medical School）
Innovative Investigation of Native and Artificial Hip Kinematics Using Dual Fluoroscopic Imaging System　…他

《神経内科で診る認知症のある疾患》

9 筋疾患の身体症状と認知症状

久我 敦* | 戸田達史*

ポイント

- 筋疾患には筋力低下・筋萎縮などの身体症状に認知症状を合併するものがある.
- 近年，筋疾患の病態の解明が急速に進展したことで，認知症状を合併するメカニズムも明らかになりつつある.
- 筋疾患に合併する認知症状の特徴を知ることは日常診療に有用である.

キーワード デュシェンヌ型/ベッカー型筋ジストロフィー，福山型筋ジストロフィー，筋緊張性ジストロフィー，知能障害

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はじめに

筋疾患の身体症状の基本は近位筋優位の筋力低下と筋萎縮であるが，筋疾患の一部には認知症状が合併する．骨格筋は中枢神経の最終効果器であるから，身体症状と認知症状はずいぶんかけ離れているように思える．しかし，両者が合併するメカニズムには各疾患の病態が深くかかわっており，その理解は日常臨床においても有用であると思われる．

本稿では，その代表的疾患としてデュシェンヌ型／ベッカー型筋ジストロフィー，福山型筋ジストロフィー，筋強直性ジストロフィーを取り挙げる．

◉認知症状を合併する筋疾患

1．デュシェンヌ型/ベッカー型筋ジストロフィー

デュシェンヌ型筋ジストロフィー（Duchenne muscular dystrophy：DMD）は3,000出生男児に1人の頻度でみられるX染色体劣性遺伝の進行性筋疾患である．筋力低下の進行は早く，乳幼児期に歩行の獲得が遅い，転びやすいといった症状が始まり，10歳代に車いす，20歳以降は人工呼吸管理を必要とする．現時点で確立した治療法はないが，原因遺伝子ジストロフィンの変異を是正するエクソン・スキッピング治療の臨床治験が進行中である．

Duchenneは最初に診たDMD症例を「脳性麻痺と偽性（筋）肥大の共存」という視点で報告しており，この症例は知的障害を合併していたと推察されている[1]．ポリオと筋ジストロフィーの鑑別すら困難であった19世紀半ばにおいて[1]，同じDMDでも知的障害を合併する場合と，しない場合があることは非常に理解しづらい現象であったにちがいない．では21世紀の我々はその理由を正確に説明できるだろうか？

まずDMDに合併する知的障害の臨床的特徴は以下のようにまとめられる．その特徴とはすなわち，①筋力低下の重症度と知的障害の重症度は相関しない，②知的障害は進行性の経過をとらない，③IQの低下は－1SDほどの軽症のものからIQが50以下というsevere mental retardationまでかなりの個人差がある，④IQのなかでも特に言語性IQが低下していること，の4点である．Cottonらは DMD患者の知能に関する32

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の臨床研究の meta-analysis を行った[2]．それによると 1,146 名の DMD 患者の IQ 測定結果の解析で，Full-scale IQ<70 の患者が全体の 34.8% を占めた．その一方で 5.9% の患者の IQ は 110 を超えており，平均以上の知能を有する患者も存在することが確認された．これまでの報告では脳重量や脳室拡大と知的障害合併の関係は判然としない．また病理学的所見に疾患特異的といえるものはない．

1987 年 Kunkel らによってジストロフィン遺伝子がクローニングされてから，ジストロフィン遺伝子の変異部位と知的障害の合併の間に何らかの規則性を見出そうとする研究が始まった．すると 5´ 側に変異がある症例と較べて 3´ 側に変異がある症例のほうが知的障害を合併しやすい傾向が明らかになった．ジストロフィン遺伝子は 79 のエクソンからなる全長 2.3Mb の巨大な遺伝子であるが，発現する組織や発生の時期によって，複雑なスプライシング調節を受けている．中枢神経系に発現するジストロフィンでは少なくとも 7ヵ所のプロモーター領域があることが確認されており，主なアイソフォームとして分子量の大きい Dp427 と分子量の小さい Dp140，Dp116，Dp71 がある（**図 1a**）．最近の研究では特に Dp140 と Dp71 の発現に影響を与える変異が知的障害の合併と関連していることが明らかとなってきている[3,4]．特に Dp71 はジストロフィン蛋白の C 末端ドメインのみから作られる．したがって，N 末端側でアクチンと結合するという骨格筋全長ジストロフィンとは機能が大きく異なると考えられている．これらの知見は「筋力低下の重症度と知的障害の重症度は相関しない」という事実ともよく合致している（**図 1b**）．

近年 DMD 患者の脳を対象とした機能的画像検査の研究も行われており PET を用いた検討では健常者と比べて右中心後回など 5 つの領域で FDG の取り込み低下が確認された[5]．これらの領域で発現しているジストロフィンのアイソフォームは何か？　そのアイソフォームの生理的機能は何か？　ということが今後さらに検討されていくであろう．

ベッカー型筋ジストロフィー（Becker muscular dystrophy：BMD）は DMD とは異なり，多くは in frame の変異であるためジストロフィン蛋白は完全には欠失していない．しかし筋力低下が軽度な BMD でもこれまで述べてきた理由から知的障害が強い場合がある（**図 1c**）．BMD は神経内科ではなく，不整脈や心不全で循環器科のみに通院していることもある．原因不明の心筋症患者で，意思疎通が困難であったり，服薬コンプライアンスが悪いケースでは BMD の可能性を考慮する必要がある．

2．福山型筋ジストロフィー

福山型筋ジストロフィー（Fukuyama congenital muscular dystrophy：FCMD）はわが国では DMD に次いで 2 番目に多い小児の筋ジストロフィーである．生後早期から哺乳力の低下などの症状があり，終生歩行を獲得することはない．多小脳回を典型とする脳奇形を合併して，重篤な知的障害を必発する．FCMD は 1959 年わが国の福山によって初めて報告されたが，当時は知的障害を伴う筋疾患という概念自体が世界でも類をみないものであった[6]．

1998 年に原因遺伝子 fukutin が同定され，fukutin 遺伝子の 3' 非翻訳領域に存在するレトロトランスポゾン挿入変異が日本人に特異的にみられるものであることがわかった（ヘテロ保因者は日本人の約 90 人に 1 人と高率である）[7]．つまり，わが国の FCMD は遺伝的に単一な疾患であり，DMD/BMD のようなアリル異質性は生じないので，知的障害が必発するのである．

ところで FCMD の分子病態は α ジストログリカン（α-dystroglycan：αDG）という糖蛋白質を中心に説明される．αDG は基底膜側の構成成分ラミニンと結合することで骨格筋の物理的強度を保証しているが，この結合には αDG に複雑な糖鎖修飾が施される必要がある．詳細は筆者らの総説[8]をご参照いただきたいが，FCMD においてはこの糖鎖修飾に異常が生じた結果，αDG がラミニンと結合できなくなり，筋ジストロフィーを発症する．FCMD の原因遺伝子 fukutin の生理的機能は現在のところ不明だが，糖鎖修飾に関

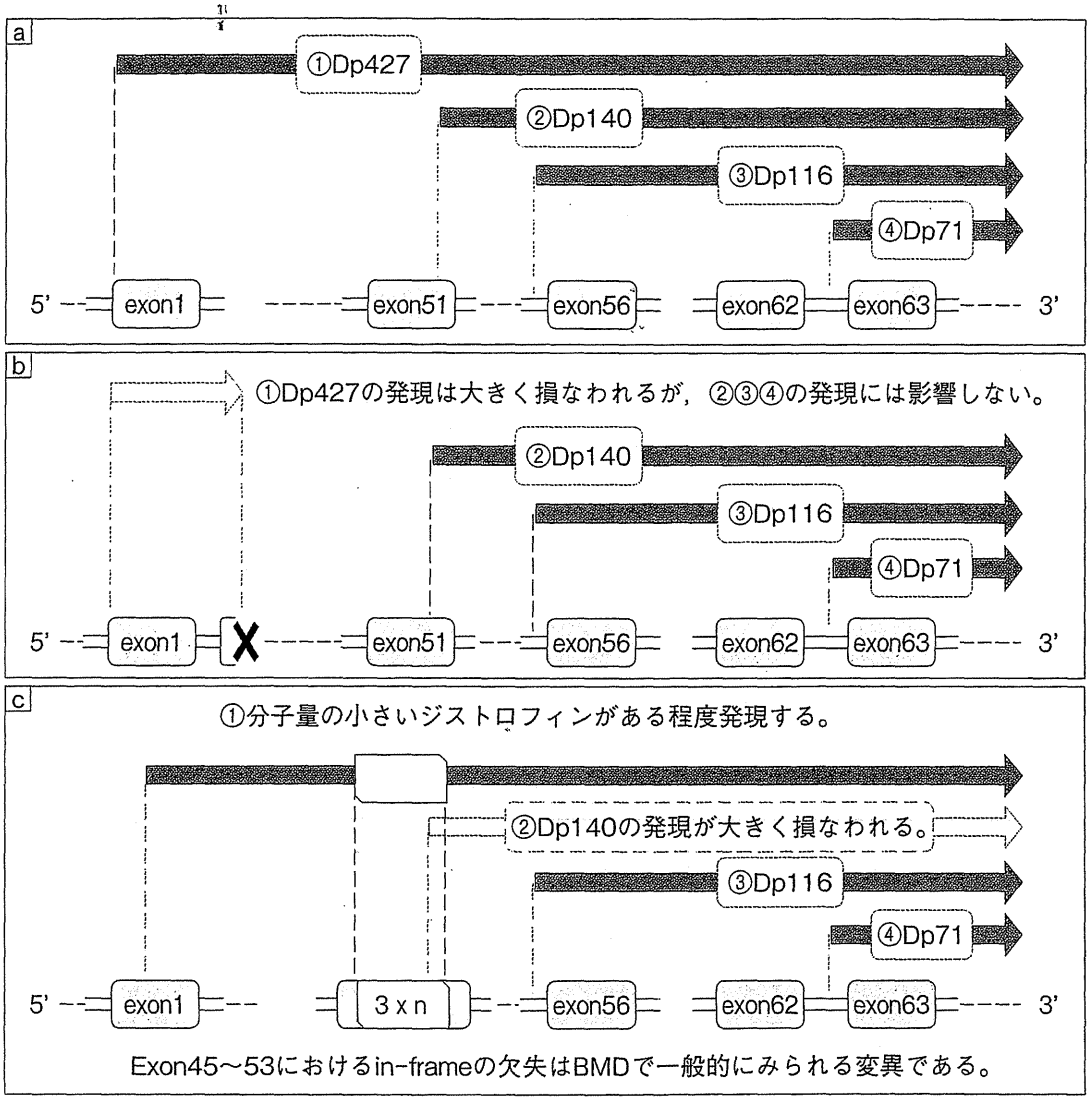

図 1　ジストロフィンアイソフォームの発現と筋症状/認知症状の関係

a：正常例
① 全長ジストロフィン Dp427 は中枢神経系でも発現しているが，骨格筋でより重要な機能を果たしている．
② Dp140 の発現調節は複雑で，プロモーター領域は intron44 に存在しており，exon45～51 の 5′非翻訳領域の影響も受けている．
③ Dp116 は成人の末梢神経に発現しているが，胎生期には脳でも発現しているので，知的障害との関係がある．
④ Dp71 は全長ジストロフィンの C 末端側にあたる部分だけで構成される．
註．網膜にのみ発現するアイソフォーム Dp260 は省略した．
b：筋症状が重篤でも知的障害を合併しない例
c：Becker 型でも知的障害が重篤である例

与する何らかの機能を有していると推測されている．

αDG は骨格筋だけでなく脳でも豊富に発現しており，多小脳回を基本とする脳病変の発生においても αDG の糖鎖修飾異常が病態の中心であると考えられる．通常，大脳皮質の表面はグリア境界膜―基底膜複合体によって覆われており，神経組織が露出することはない．しかし FCMD では，基底膜の構成成分ラミニンとの結合能が低下するため，グリア境界膜―基底膜複合体に破れが生じ，胎生期の神経細胞の移動を阻止できずに神経組織がくも膜下腔へ迷出し，多小脳回が発生するものと考えられる．

表 1　筋強直性ジストロフィーの臨床症状

・中枢神経系	精神発達遅延～知能低下，日中の傾眠，情緒障害
・筋骨格系	筋力低下，筋萎縮，ミオトニア
・循環器系	心伝導ブロック，不整脈，心筋症
・消化器系	消化管運動障害
・呼吸器系	呼吸不全
・内分泌系	耐糖能異常，女性化乳房，性腺機能不全
・血液系	低ガンマグロブリン血症
・その他	白内障，前頭部禿頭

さらに近年，fukutin 遺伝子の点変異症例は古典的 FCMD と異なる臨床像をとることがわかってきた．点変異のホモ接合あるいは点変異とレトロトランスポゾン挿入変異の複合ヘテロ接合の場合，その点変異の影響の大きさによって重症度が変化する．一般に複合ヘテロ接合の患者は重症であることが多いが，最近ではむしろ軽症で知的障害を合併しない症例の報告もあり，注目されている[9,10]．

3．筋強直性ジストロフィー

筋強直性ジストロフィー（Myotonic dystrophy：DM）の特徴として**表 1** に挙げた多彩な身体症状がある．DM は筋原性疾患ではないので，その筋症状と認知症状は全身性疾患の一症状と理解することができる．

DM の原因遺伝子には 2 種類あって，わが国では DMPK 遺伝子が原因の DM type 1（DM1）が大多数である．しかし，最近になって ZNF9 遺伝子が原因の DM type 2（DM2）がわが国にも存在することが報告された[11]．DM1，DM2 は共に常染色体優性遺伝形式をとる．DM1 では 3´ 非翻訳領域内で CTG リピートが，DM2 では最初のイントロン内で CCTG リピートが異常伸長している．患者家系では世代を経るに従ってリピート数が増加するため，発症年齢が低下して重症化する現象（anticipation）が観察される．この現象は特に女性 DM 患者から子供へ遺伝する場合により顕著である．

DM ではリピート数の増加と相関して IQ が低下する[12]．リピート数が数千まで伸長すると著明な筋力低下に精神発達遅延を合併する congenital form となる（註．DM2 には congenital form はない）．たとえば産婦人科から「フロッピーインファント」に関してコンサルトを受けて NICU へ行くと，母親が診断未確定の DM 患者であった，というような事態に遭遇すると，anticipation という現象の深刻さを痛感させられるものである．Congenital form あるいは小児発症例では精神発達遅滞に加えて，自閉症やアスペルガー症候群，注意欠陥障害などの範疇で捉えられる症状の合併も少なくない．

一方で，内科を受診する DM 患者の多くは成人発症である．成人 DM 患者の日常臨床で問題になる認知症状は，IQ の低下といった数値では表現しづらい独特のものである．本症患者と接する医療者誰もが診療上困難を感じるのは，特有の「コミュニケーションのとりづらさ」ではないだろうか．わが国の小早川，河村らの検討[13]によると DM1 患者ではネガティブな情動（怒り，嫌悪，恐怖など）を示す表情の認知が障害されており，コミュニケーション障害の要因の一つになっている可能性がある．

最近の研究によって，DM1 患者では CUG リピートが延長した DMPK1 転写産物によって RNA 結合蛋白の機能が障害され，さまざまな遺伝子の pre-mRNA にスプライシング異常が生じることがわかってきた．たとえば病名の由来であるミオトニアの原因としては骨格筋の Cl チャンネル遺伝子のスプライシング異常が考えられており，動物モデルでも実証されている．あるいは耐糖能異常に関して，インスリン受容体遺伝子のスプライシング異常が示されている．

認知症状の原因として，脳に発現する遺伝子のなかでもっともよく研究されているのがタウ遺伝子のスプライシングの変化である．リピート数の増加に従ってタウの胎児型アイソフォームの発現量が増えることが実証されており，病態との関連が推測されている．タウ蛋白はアルツハイマー病の病理所見である神経原線維性変化のなかに見出されることで有名で，またタウ遺伝子の変異に

よって前頭側頭葉型認知症が生じる．これらはDM患者の脳において，脳室拡大と脳萎縮を認めること，病理所見としてアルツハイマー病と同じく神経原線維変化を認めることとよく合致している．

まとめ

筋ジストロフィーは遺伝子の異常にアプローチする治療の対象疾患として今後さらに注目を集めると考えられる．本稿で述べた疾患はいずれも筋症状に認知症状を合併しうる．合併する背景にある分子病態の理解が今後の遺伝子診療には不可欠であると思われる．

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症例報告

骨格筋でのみアミロイドの沈着を確認しえたアミロイドーシスの1例

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要旨：症例は75歳男性，7カ月前から進行する歩行障害を主訴に入院した．両下肢近位筋優位の四肢筋力低下と腱反射低下をみとめた．血清免疫電気泳動検査でIgAλ型M蛋白が陽性，骨髄中に形質細胞の単クローン性増加をみとめIgA型多発性骨髄腫と診断した．CTにて異常がみられた外側広筋の筋生検ではring fiber様の異常筋線維をみとめ，その細胞質周囲はCongo redで染色され，アミロイドミオパチーと診断した．多発性骨髄腫にともなう貧血・腎障害・骨病変などの臓器障害はみとめず，心筋・直腸粘膜および腓腹神経の生検ではアミロイドの沈着を証明できなかった．本例は骨格筋においてのみ証明しえたアミロイドーシスであった．

（臨床神経 2012;52:739-743）
Key words：アミロイドミオパチー，アミロイドーシス，多発性骨髄腫

はじめに

アミロイドーシスとは不溶性の線維蛋白質であるアミロイドが諸臓器に沈着することによって生じる疾患群である．神経系では末梢神経へのアミロイド沈着が多く，骨格筋への沈着はまれである．今回，われわれは骨格筋生検によりアミロイドの沈着を確認しえたアミロイドミオパチーの症例を経験した．アミロイドーシスでは多臓器が障害され，各臓器での生検にてアミロイドの沈着が確認されることも多いが，本例ではアミロイドの沈着は骨格筋のみに限局して証明された．

症　例

症例：75歳　男性
主訴：歩行障害
既往歴：10代後半から慢性C型肝炎，68歳より高血圧，70歳より肥大型心筋症と診断され利尿薬を投与されていた．
家族歴：特記事項なし．
現病歴：2008年9月より両下肢の脱力感が出現し，階段を昇りづらくなった．同12月から手をつかないと床から立ち上がれなくなった．2009年1月に他院にて血清中CKが500 IU/l台の上昇を指摘された．同3月から歩行の際に杖が必要になった．同4月，筋疾患がうたがわれ当科に紹介受診した．
一般身体所見：両下腿にpitting edemaをみとめた．
神経学的所見：意識清明で，高次機能や脳神経領域に異常はなかった．四肢にMMT3～4/5程度の下肢優位，近位筋優位の筋力低下をみとめた．筋萎縮や筋肥大はみとめなかった．四肢腱反射はすべて低下しており，両下肢で振動覚が低下していた．
検査所見：一般検血に異常はなかったが，生化学検査でCK 519IU/lと上昇していた．抗核抗体，抗Jo-1抗体，抗U1-RNP抗体など自己抗体は陰性であった．IgG 651mg/l，IgM 29mg/lと低下，IgA 1,130mg/dlと上昇していた．血清免疫電気泳動検査ではIgAλ型M蛋白陽性，尿中λ型Bence-Jones蛋白も陽性であった．骨髄穿刺で，形質細胞の単クローン性増加をみとめたためIgA型多発性骨髄腫と診断した．
筋CTでは両側下肢筋に低吸収域をみとめ，広範な脂肪置換がうたがわれた．上肢の筋には明らかな異常はみとめられなかった（Fig. 1）．運動神経伝導検査では両側正中神経で遠位潜時の延長（右5.2ms，左4.3ms）をみとめた．感覚神経伝導検査では左正中神経の感覚神経活動電位（sensory action potential，SAP）が8.0μVと低値で，右正中神経のSAPや両側腓腹神経のSAPは導出されなかった．針筋電図検査では右上腕二頭筋・右大腿直筋・右三角筋でfibrillationやpositive sharp wave（PSW），低振幅，短持続のmotor unit potentials（MUP）をみとめ，早期動員パターンを呈していた．
以上の所見から，骨髄腫にともなった筋障害と遠位優位の末梢神経障害の合併をうたがった．筋障害の確定診断の目的で左外側広筋から筋生検をおこなった．ヘマトキシリン・エ

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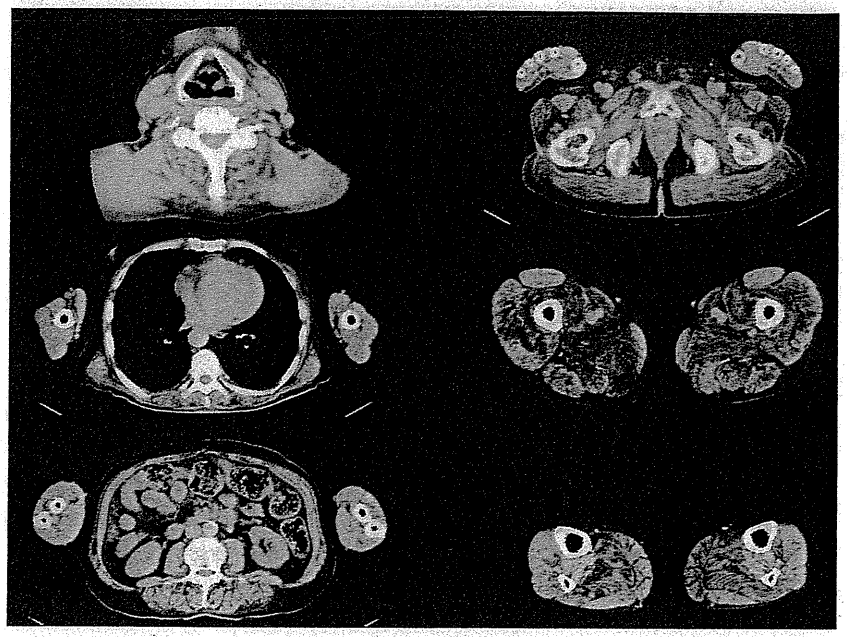

Fig. 1 Muscle CT scan images.
Muscle CT scan showed severe fatty infiltration of femoral muscles. Moderate fatty infiltration was observed in gastrocnemius, soleus, and tibialis anterior muscles.

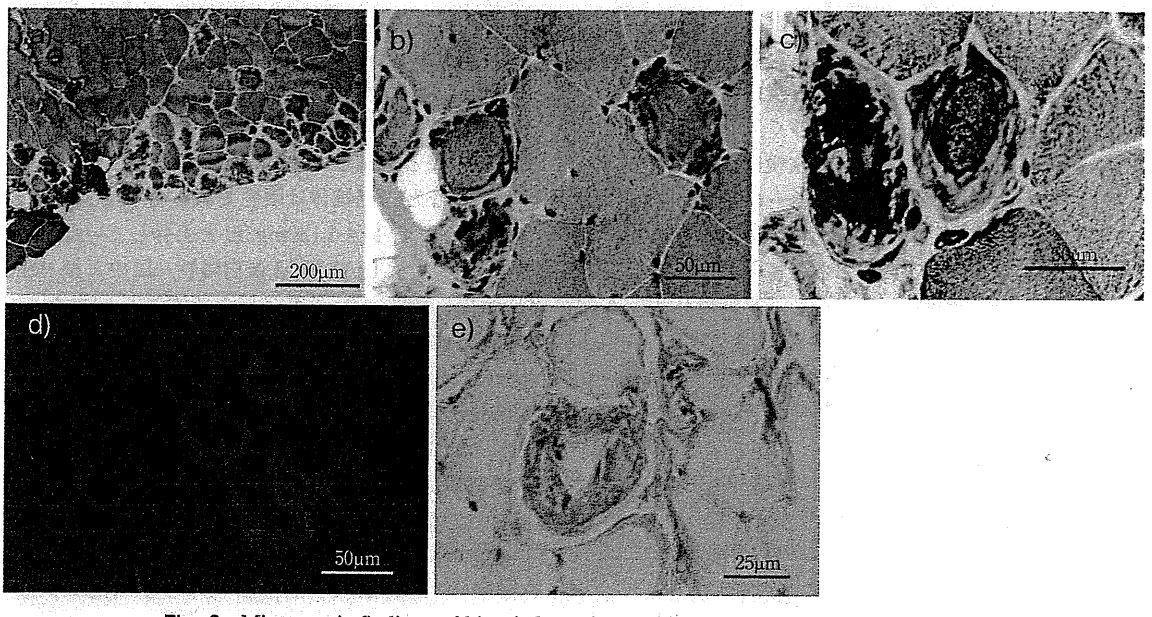

Fig. 2 Microscopic findings of biopsied specimen of left vastus lateralis muscle tissue.
a), b) Hematoxylin and eosin stained cryosections showed peripheral replacement of cytoarchitecture with basophilic material and a preserved core ringed with nuclei in some fibers.
c) NADH cryosections showed disorganized intermyofibrillar networks at the periphery of abnormal fibers.
d) Congo red-stained cryosections viewed under a fluorescence microscope demonstrates marked congophilia of endomysium.
e) Amyloid deposits were stained by anti-λ light chain antibody (detection by DAB).

オジン（HE）染色では，著明な筋線維の大小不同に加えて，ring fiber 様に辺縁部が好塩基性に染色され多数の核を有する筋線維をみとめた．これらの筋線維の周辺部は NADH 活性を欠いているか，筋原線維の配列がいちじるしく乱れていた．異常筋線維の細胞質周囲，ならびに一部の間質は Congo red で染色されるアミロイドの沈着をみとめ，アミロイドミ

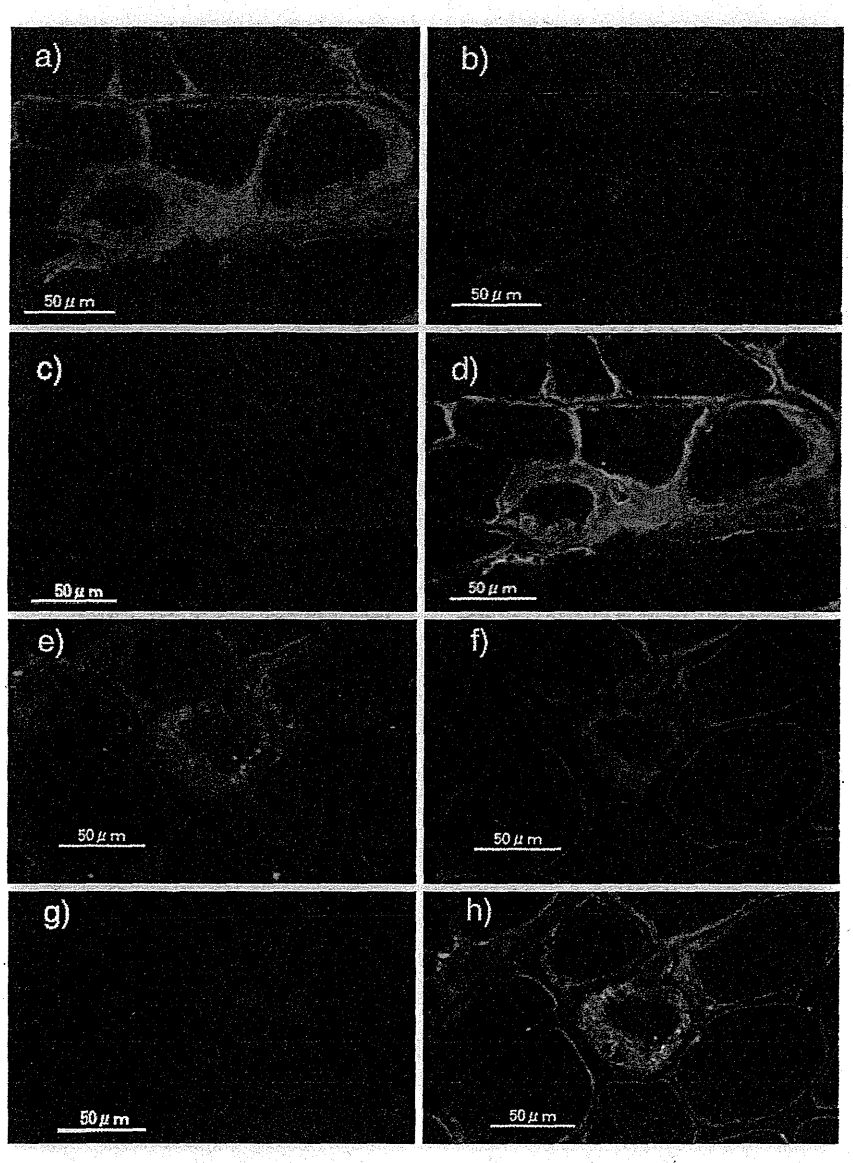

Fig. 3　Double immunohistochemical staining of left vastus lateralis muscle tissue using anti-dystrophin antibody and anti-λ light chain antibody (green: anti-λ light chain antibody, red: anti-dystrophin antibody, blue: DAPI).
a)-d) Anti-λ light chain antibody-positive sites were present outside of the anti-dystrophin antibody positive sites. Deposition of amyloid on the outside of the cell membrane was indicated.
e)-h) In some muscle fibers, the anti-dystrophin antibody and anti-λ light chain antibody positive area looked like a thick layered membrane. Many nuclei were observed on the periphery of these muscle fibers.

オパチーと診断した．抗λ鎖抗体をもちいた免疫染色でアミロイド沈着部分に抗λ鎖抗体陽性所見をみとめ，ALアミロイドの沈着であることを確認した(Fig. 2)．またアミロイドの沈着部位と細胞膜との位置関係を確認するため抗ジストロフィン抗体と抗λ鎖抗体との二重免疫染色をおこない，筋細胞膜より外側にアミロイドが沈着していることを確認した．また一部の筋線維では筋細胞膜が重なるように厚くなり，同部位にアミロイドが沈着していた(Fig. 3)．末梢神経障害合併の有無を確認するため，左腓腹神経生検を施行したが軽度の有髄線維密度の低下をみとめるのみで，アミロイドの沈着はみとめなかった．全身検索の目的で，直腸粘膜生検や心筋生検もおこなったが，これらの組織にはアミロイドの沈着はみとめられなかった．

考　察

アミロイドーシスとはβシート構造を有する蛋白質の立体構造のミスフォールディングと重合により生じた不溶性の線維蛋白質であるアミロイドが諸臓器に沈着することによって臓器障害をきたす疾患群である．アミロイドーシスの原因と

しては原発性または多発性骨髄腫にともなうALアミロイドーシスや家族性アミロイドーシスなどが知られている．アミロイドの沈着臓器としては腎臓，心臓，肝臓，末梢神経などが多く，筋組織への沈着はまれとされており，Praysonらの報告によると生検筋3,937例のうちアミロイド沈着をみとめたのは16例（0.4%）に過ぎなかった[1]．

一般にアミロイドミオパチーは中高齢の男性に多く，下肢近位筋優位の筋力低下を呈し，筋の仮性肥大による巨舌やヘラクレス体型が特徴的所見として知られている[2][3]．しかしChapinらの報告によると，仮性肥大がみとめられたのはアミロイドミオパチー79例中27例で[4]，Gertzらの報告でも12例中3例のみと[5]，その出現頻度は決して高いとはいえず，本例のように仮性肥大がみとめられない症例も多い．中高齢者の原因不明の筋障害のばあい，仮性肥大がみとめられなくともアミロイドミオパチーを鑑別にあげる必要がある．

検査所見では血清CKは正常ないし上昇しても軽度にとどまることが多い．血清中もしくは尿中にM蛋白をみとめることは，アミロイドーシスの原因疾患を診断する上で重要な所見である．針筋電図では安静時放電をともなう筋原性変化を呈する．筋病理所見ではアミロイドの沈着が筋周膜や筋内膜・血管周囲にみとめられる．本例ではHE染色でring fiber様に周囲が好塩基性に染色され，辺縁部に多数の核を有する異常筋線維をみとめており，Chapinらの報告[4]と比較しても，アミロイドミオパチーに特徴的な病理所見と考えられる．しかし異常筋線維の周囲にみられた核は筋鞘核か他の細胞成分の核かなのかは不明であり今後，検討すべき課題である．筋原性変化に加えて小角化線維の集簇や筋線維タイプ群化といった神経原性変化をみとめることが多く，Prayson ら[1]はアミロイドミオパチーで腓腹神経生検をおこなった7例のうち，全例で末梢神経にアミロイドを確認している．筋病理所見において高頻度に神経原性変化が混在しているのはアミロイドミオパチーを発症する時点ですでにアミロイドニューロパチーをきたしている症例が多いためと考えられる．本例では神経伝導検査では末梢神経障害の存在が示唆され，筋生検でも一部に小角化線維の集簇をみとめた．腓腹神経生検では軽度の有髄線維密度の低下をみとめ，末梢神経障害の原因がアミロイドーシスである可能性は否定できないがCongo red染色は陰性であり，アミロイドは同定されなかった．また直腸粘膜・心筋の生検でもアミロイドは同定されなかった．

アミロイドミオパチーにおける骨格筋の機能障害の機序としては，筋線維の直接的な障害が原因とする説やアミロイドが血管に沈着することで筋細胞が虚血に陥ることによるとする説がある[5]．アミロイドミオパチーの症例において生検筋の電子顕微鏡による観察でアミロイドフィラメントに取りかこまれた筋の筋細胞膜・基底膜の破壊をみとめたことが報告されている[6]．われわれの症例では，抗λ鎖抗体による免疫染色で筋細胞の周囲にアミロイドが沈着している像が確認された．また筋細胞膜が重なるように変化している像もみとめられ，アミロイドによる筋細胞膜への直接的な障害の可能性が示唆された．

本例は多発性骨髄腫にともなうアミロイドミオパチーであるが，貧血・腎障害・骨病変など典型的な臓器障害をみとめず，通常診断に有用とされる直腸粘膜生検や神経生検ではアミロイドの沈着を証明できなかった．そのため筋力低下の原因特定のために筋生検をおこない，はじめてアミロイドーシスの診断が確定した．筋障害の所見があり，他の臓器においてアミロイドーシスを示唆する所見が乏しいばあいは，積極的に筋生検をおこなうべきである．

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※本論文に関連し，開示すべきCOI状態にある企業，組織，団体はいずれも有りません．

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Abstract

A case of amyloidosis with amyloid deposition detected only in skeletal muscles

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A 75-year-old man was admitted to our hospital with progressive weakness in the lower extremities for 7 months. Immunoelectrophoresis of serum detected IgA λ type M protein and bone marrow examination detected an increase in monoclonal plasma cells, thus leading to a diagnosis of IgA λ type multiple myeloma. Subsequent muscular CT scan showed severe fatty infiltration of vastus lateralis muscles, and histopathological examinations of biopsied muscle specimens an abundance of abnormal "ring-fiber-like" appearance, positive staining by Congo red and the presence of anti-λ light chain antibody. This led to a diagnosis of amyloid myopathy. No depositions were seen in rectal mucosa, cardiac muscle, or sural nerve. The results of double immunohistochemical staining using anti-dystrophin antibody and anti-λ light chain antibody suggested the possibility of direct injury by amyloid to muscle fibers. The case presented here was thus amyloidosis confirmed by deposition of amyloid only in muscles. In conclusion, when amyloidosis is suspected and there is evidence of muscle injury, muscle biopsy should be performed.

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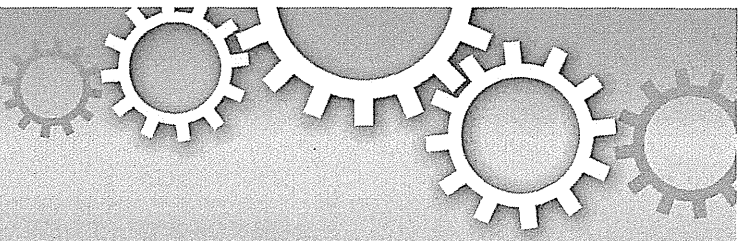
Key words: amyloid myopathy, amyloidosis, multiple myeloma

SCIENTIFIC REPORTS



SUBJECT AREAS:
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PINK1-mediated phosphorylation of the Parkin ubiquitin-like domain primes mitochondrial translocation of Parkin and regulates mitophagy

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Parkinson's disease genes *PINK1* and *parkin* encode kinase and ubiquitin ligase, respectively. The gene products PINK1 and Parkin are implicated in mitochondrial autophagy, or mitophagy. Upon the loss of mitochondrial membrane potential ($\Delta\Psi$m), cytosolic Parkin is recruited to the mitochondria by PINK1 through an uncharacterised mechanism – an initial step triggering sequential events in mitophagy. This study reports that Ser65 in the ubiquitin-like domain (Ubl) of Parkin is phosphorylated in a PINK1-dependent manner upon depolarisation of $\Delta\Psi$m. The introduction of mutations at Ser65 suggests that phosphorylation of Ser65 is required not only for the efficient translocation of Parkin, but also for the degradation of mitochondrial proteins in mitophagy. Phosphorylation analysis of Parkin pathogenic mutants also suggests Ser65 phosphorylation is not sufficient for Parkin translocation. Our study partly uncovers the molecular mechanism underlying the PINK1-dependent mitochondrial translocation and activation of Parkin as an initial step of mitophagy.

Mutations of the *PINK1* gene cause selective degeneration of the midbrain dopaminergic neurons in autosomal recessive juvenile Parkinson's disease (PD)[1]. The *PINK1* gene encodes a serine/threonine kinase with a predicted mitochondrial target sequence and a putative transmembrane domain at the N-terminus[2–5]. Loss of the *PINK1* gene in *Drosophila* results in the degeneration of mitochondria in cells with high energy demands, such as muscle and sperm cells, which is suppressed by the introduction of the *parkin* gene, another gene responsible for autosomal recessive juvenile PD[6–8]. The gene product Parkin encodes a RING-finger type ubiquitin ligase (E3) with a Ubl domain at the N-terminus[9–12].

A series of cell biological studies have provided strong evidence that there are important roles for PINK1 and Parkin in regulating mitochondrial homeostasis. PINK1 is constitutively proteolysed by the mitochondrial rhomboid protease, PARL, at the mitochondrial membrane of healthy mitochondria, resulting in processed forms of PINK1[13–16]. The processed PINK1 is rapidly degraded by the proteasome[2,17]. The reduction of $\Delta\Psi$m leads to the accumulation and activation of PINK1 in the mitochondria[17–19] through a currently unresolved mechanism[20]. The accumulation of PINK1 recruits Parkin from the cytosol to the mitochondria with decreased membrane potential, which stimulates Parkin E3 activity, promoting mitochondrial degradation via an autophagic event known as mitophagy[17,21–24]. The recruitment of cytosolic Parkin to the mitochondria upon disruption of $\Delta\Psi$m is believed to be the first step of mitophagy for the removal of damaged mitochondria. This recruitment is required for the kinase activity of PINK1[17,21–25]. Although two separate studies have proposed that Parkin is directly phosphorylated by PINK1[26,27], others have failed to detect Parkin phosphorylation by PINK1[21], suggesting that the kinase activity of PINK1 itself is relatively low. One reason biochemical analysis has been unable to obtain direct evidence is that recombinant human PINK1 purified from mammalian cultured cells or bacteria easily loses kinase activity, while insect PINK1 has significant autophosphorylation activity[28,29].

Very recently, Kondapalli, C. *et al.* reported that PINK1 directly phosphorylates Parkin at Ser65 in the Ubl domain[18]. However, the extent and consequences of Parkin phosphorylation by PINK1 in mitochondrial regulation are still not fully understood.

To address this issue, we attempted to independently monitor and compare the phosphorylation status of Parkin in wild-type and *PINK1*-deficient cells, thereby excluding the possibility of phosphorylations by uncharacterised kinases other than PINK1[30]. Here, we also report that Parkin is demonstrably phosphorylated at Ser65 in a PINK1-dependent manner. Furthermore, we show that this phosphorylation event is implicated in the regulation of mitochondrial translocation of Parkin and the subsequent degradation of mitochondrial surface proteins during mitophagy.

Results

Parkin is phosphorylated upon depolarisation in ΔΨm. We used [^{32}P] orthophosphate to metabolically label mouse embryonic fibroblasts (MEFs) derived from *PINK1* deficient mice, in which HA-tagged Parkin together with FLAG-tagged wild-type or kinase-dead forms (triple mutant with K219A, D362A and D384A) of PINK1 were virally introduced (hereafter referred to as "PINK1-FLAG WT" or "KD/HA-Parkin/*PINK1*$^{-/-}$" MEFs) and then induced Parkin-mediated mitophagy via treatment with the protonophore carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP). As shown in Figure 1a, Parkin was specifically phosphorylated in CCCP-treated PINK1-FLAG WT/HA-Parkin/*PINK1*$^{-/-}$ MEFs, but not in PINK1-FLAG KD/HA-Parkin/*PINK1*$^{-/-}$ MEFs. Phos-tag Western blotting, in which phosphorylated proteins appear as slower migrating bands[28], revealed that Parkin was phosphorylated within 10 min following CCCP treatment (Fig. 1b). Phosphorylation of Parkin reached its maximum level approximately 40 min after CCCP treatment and was sustained at least until 6 hr (Supplementary Fig. S1). Under these conditions, slower migrating bands of PINK1 also appeared, which very likely reflects the autophosphorylation of PINK1 when activated (Fig. 1b)[18]. The suppression of PINK1 accumulation by RNA interference suggested thatΔΨm depolarisation-dependent activation of PINK1 along with PINK1 accumulation is a key element for Parkin phosphorylation (Fig. 1c). Every PINK1 deletion and pathogenic mutant we tested failed to stimulate Parkin phosphorylation effectively, strongly suggesting that intact PINK1 is required for this action (Fig. 1d and e). Importantly, human fibroblasts from a patient with *PINK1*-linked parkinsonism also lacked the activity to phosphorylate Parkin (Fig. 1f). The phosphorylated Parkin disappeared within 30 min during the recovery of ΔΨm depolarisation by the removal of CCCP from the culture medium (Fig. 1g). Further analysis using phosphatase and proteasome inhibitors suggested that phosphorylated Parkin is at least partly degraded by proteasomal activity in the mitochondria (Supplementary Fig. S2).

Phosphorylation of Ser65 in the Parkin Ubl domain primes the mitochondrial translocation of Parkin. To determine which residue(s) of Parkin are phosphorylated, we immunopurified HA-tagged Parkin from PINK1-FLAG WT or KD/HA-Parkin/*PINK1*$^{-/-}$ MEFs treated with or without CCCP and performed mass spectrometric analysis for phospho-peptides (Supplementary Fig. S3). Although Phos-tag Western blotting of Parkin mainly detected a single band shift, which represents a single phospho-modification, the mass spectrometric analysis identified Ser9 or Ser10 and Ser65, Ser101 and Ser198 as phosphorylated residues of Parkin. Among these residues, only Ser65 phosphorylation increased (33-fold) in CCCP-treated PINK1-FLAG WT/HA-Parkin/*PINK1*$^{-/-}$ MEFs (Supplementary Fig. S3). Phos-tag Western blotting with mutant forms of Parkin, in which the identified phospho-serine residues are replaced with alanine, revealed that the band shift represents Ser65 phosphorylation (Fig. 2a). An *in vitro* kinase assay with recombinant insect PINK1, which has marked kinase activity[28], strongly suggested that PINK1 directly phosphorylates Parkin at Ser65 (Supplementary Fig. S4). The Ser65 residue lies in the Ubl domain and is highly conserved from human to *Drosophila* (Fig. 2b). We next examined whether phosphorylation of Ser65 is required for Parkin-mediated mitophagy. GFP-tagged Parkin WT, which was localised both in the cytoplasm and in the nuclei of mock (DMSO)-treated cells (0 hr, Fig. 2c and d), was translocated to the mitochondria and induced the perinuclear aggregation of mitochondria 2 hr after CCCP treatment, as previously reported (2 hr, Fig. 2c and d)[17,23]. Replacement of Ser65 with alanine (S65A) did not affect the subcellular localisation of Parkin in mock-treated cells when compared with that of GFP-Parkin WT (0 hr, Fig. 2c and d). However, GFP-Parkin S65A almost completely inhibited the mitochondrial translocation of Parkin and the perinuclear rearrangement of mitochondria 0.5 hr after CCCP treatment (0.5 hr, Fig. 2c and d) and showed delayed translocation in 2 hr (2 hr, Fig. 2c and d). The expression of a putative phosphomimetic Parkin S65E also showed a subcellular localisation similar to that of GFP-Parkin WT in both DMSO- and CCCP-treated cells (Fig. 2c). However, GFP-Parkin S65E exhibited a mild translocation defect, suggesting that S65E does not fully mimic the phosphorylated Ser65 (Fig. 2d).

Parkin Ser65 phosphorylation is not sufficient for mitochondrial translocation upon depolarisation of ΔΨm. As PINK1-mediated Ser65 phosphorylation appeared to be required for efficient translocation of Parkin, we next examined whether well-characterised pathogenic Parkin mutants were subjected to phosphorylation upon CCCP treatment. In this experiment, we used three kinds of Parkin mutants based on the previous and current studies (Supplementary Fig. S5)[17,22,23]. The first group, V15M, P37L, R42P and A46P, had intact or weakly impaired mitochondrial translocation activity. The second group, T415N and G430D, had mildly impaired translocation activity. The third group, K161N, K221N and T240R, almost completely lacked translocation activity (Fig. 3a). Surprisingly, all of the mutants possessed comparable phosphorylation efficiencies to those of WT (Fig. 3b). This result suggests that Ser65 phosphorylation is not sufficient for the mitochondrial translocation of Parkin.

Biochemical fractionation of endogenous Parkin from SH-SY5Y cells detected only the phosphorylated form of Parkin in the mitochondrial fraction upon CCCP treatment (Fig. 3c), which strongly suggests that phosphorylation of Parkin is required for mitochondrial translocation. There was a slight difference in the gel mobility of phosphorylated Parkin between the cytosolic and the mitochondrial fractions and between CCCP-treated periods of time. These differences very likely reflect differences in the complexity of the contents of each fraction rather than in the phosphorylation status of Parkin because a single shifted band appears in the mixed fractions (Mito + Cyto in Fig. 3c; CCCP 30 min + 60 min in Supplementary Fig. S6).

Effect of Parkin Ser65 phosphorylation on the autophagic reaction. We next examined whether Ser65 phosphorylation is required for the subsequent autophagic reaction, in which various ubiquitin-proteasome- and autophagy-related proteins are involved, including the 26S proteasome, p97/VCP, p62/SQSTM1, LC3, ATG5 and ATG7[22,23,31–35]. Parkin has been reported to be involved in the ubiquitin-proteasome-dependent degradation of a variety of mitochondrial outer membrane proteins, including Mitofusin1 (Mfn1)[32], Mfn2[32], Miro1[36,37], Miro2[37], VDAC1[22] and Tom20[31]. Degradation of Mfn1, VDAC1 and Tom20 at the mitochondrial outer membrane was observed in PINK1 WT/GFP-Parkin/*PINK1*$^{-/-}$ MEFs 1 to 4 hr after CCCP treatment (Fig. 4a). While GFP-Parkin harbouring S65A or S65E mutations was also capable of inducing Mfn1, VDAC1 and Tom20 degradation, the efficiency was impaired, especially in Mfn1 and VDAC1 (Fig. 4a). Long-term time course analysis revealed that in cells expressing Parkin with S65A or S65E mutations, Mfn1 and VDAC1 cannot be degraded effectively, and the mitochondrial outer membrane was likely more intact as indicated by the sustained

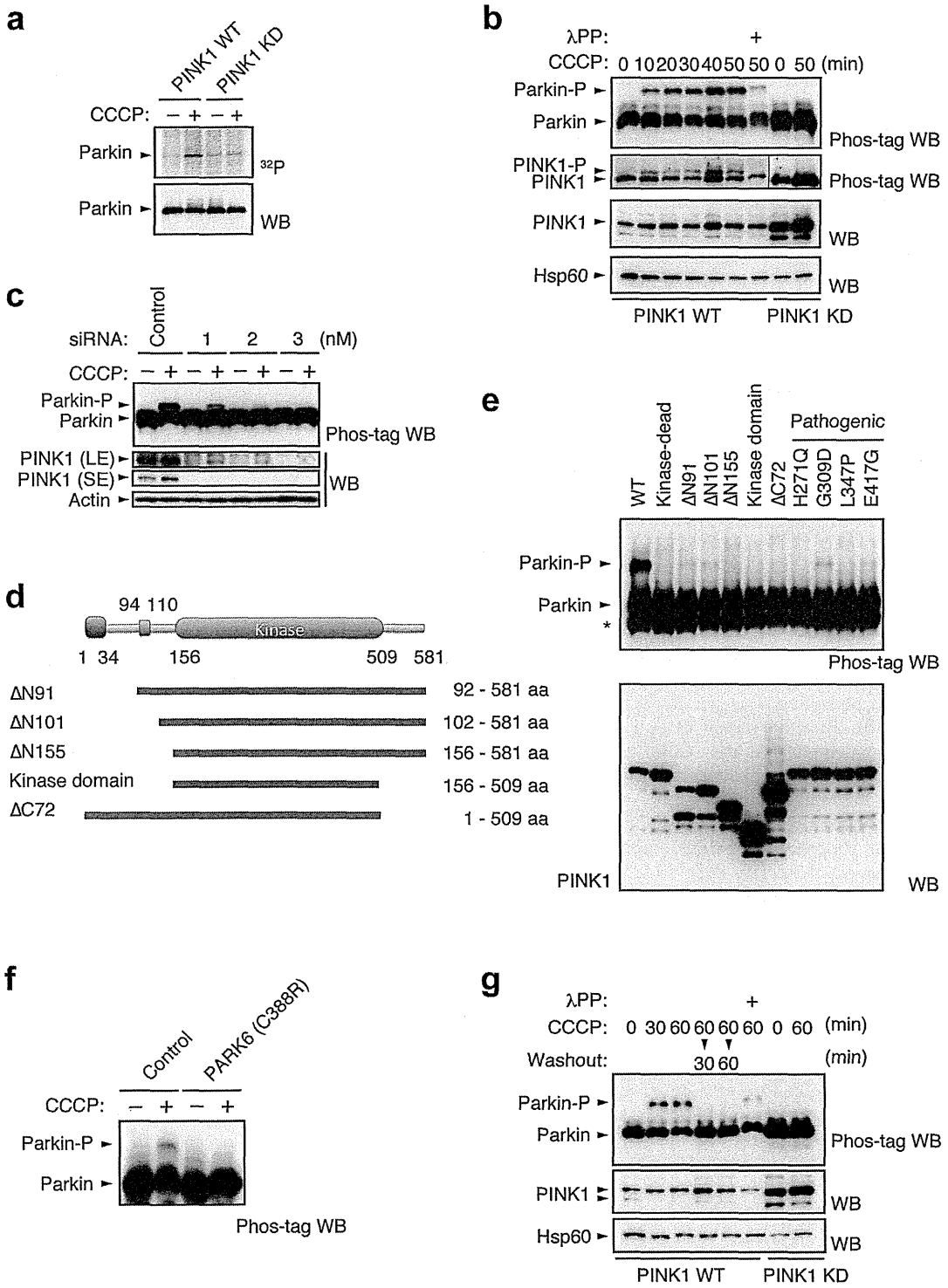

Figure 1 | PINK1-dependent phosphorylation of Parkin *in vivo*. (a) PINK1-FLAG WT or KD/HA-Parkin/*PINK1*$^{-/-}$ MEFs were labelled with [^{32}P] orthophosphate and treated with 30 μM CCCP for 1.5 hr. Phosphorylated Parkin was detected by autoradiography (^{32}P). Immunoprecipitated HA-Parkin was detected by Western blotting (WB) with anti-Parkin. (b) PINK1-FLAG WT or KD/HA-Parkin/*PINK1*$^{-/-}$ MEFs were treated with or without 30 μM CCCP for the indicated periods of time. Cell lysate was subsequently separated on a Phos-tag gel, followed by WB with anti-PINK1 or anti-Parkin antibodies (Phos-tag WB). Phosphorylated bands of Parkin and PINK1 were confirmed by their disappearance with lambda protein phosphatase (λPP) treatment. Mitochondrial Hsp60 was used as a loading control. (c) Suppression of endogenous PINK1 expression inhibits Parkin phosphorylation. HeLa cells stably expressing non-tagged Parkin were treated with the indicated concentrations of stealth siRNA duplex against PINK1 (Invitrogen) with or without 10 μM CCCP for 1 hr. Long- (LE) and short-exposure (SE) blot signals for PINK1 were shown. Actin was used as a loading control. (d) Truncated PINK1 mutants used in this study. Putative mitochondria-targeting sequence, 1–34 aa; transmembrane domain, 94–110 aa; kinase domain, 156–509 aa. (e) Full-length PINK1 is required for Parkin phosphorylation. *PINK1*$^{-/-}$ MEFs stably expressing non-tagged Parkin were transfected with various PINK1 constructs with C-terminal FLAG-tags. PINK1 expression was confirmed with anti-FLAG-HRP. (f) Human fibroblasts from a normal control and a *PARK6* case with a homozygous C388R mutation[44] were transfected with Parkin and were treated with or without 30 μM CCCP for 1 hr. (g) Cells treated with CCCP up to 60 min as in (b) were further incubated with fresh culture medium without CCCP for the indicated periods of time (Washout).

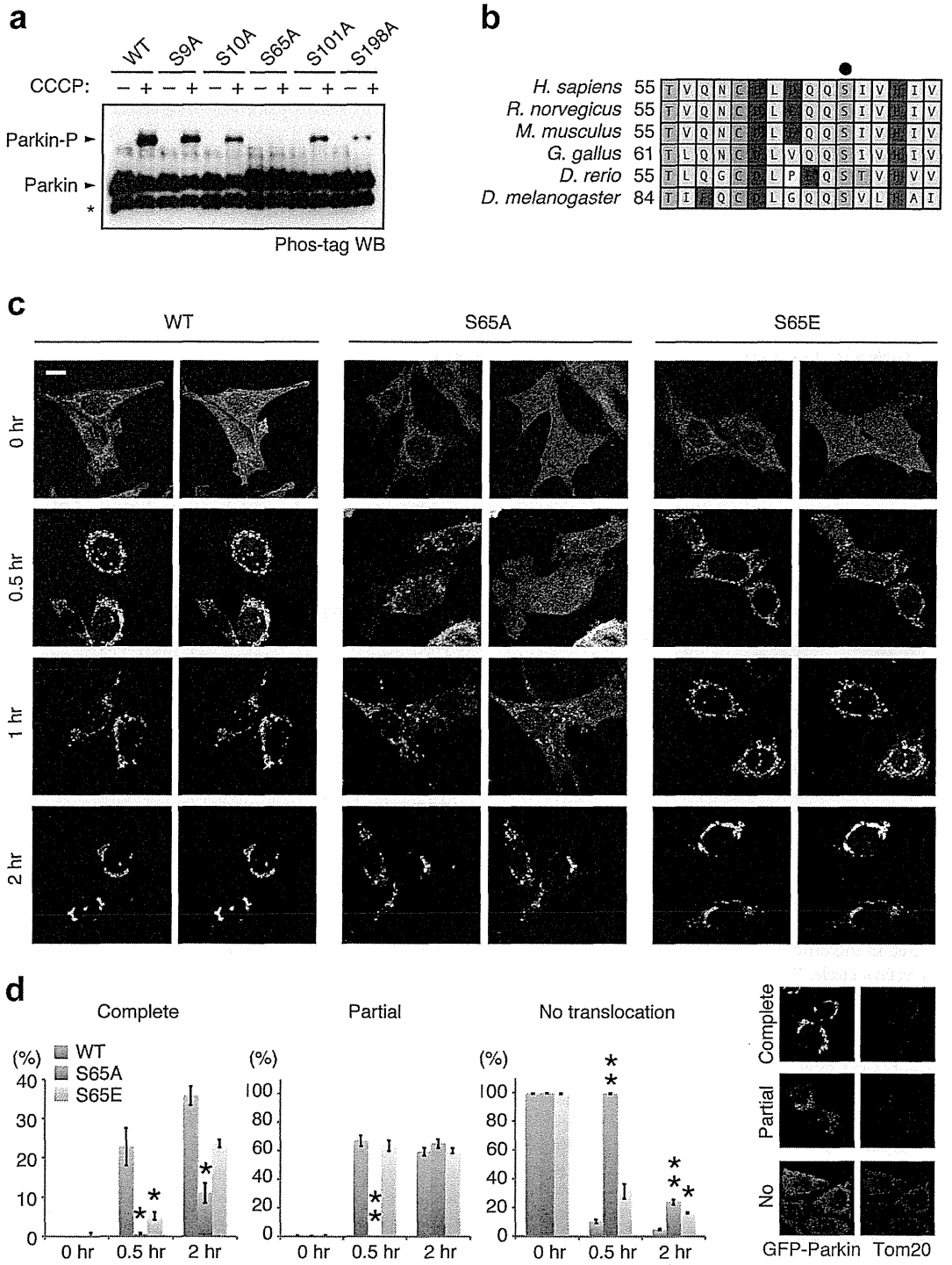

Figure 2 | Ser65 in the Ubl domain of Parkin is phosphorylated upon depolarisation of $\Delta\Psi$m. (a) Phos-tag Western blotting detected phosphorylation of Ser65. HeLa cells were transiently transfected with Parkin WT and a series of alanine mutants for the candidate phospho-residues followed by treatment with or without 20 μM CCCP for 1 hr. Cell lysates were analysed by Phos-tag Western blotting. An asterisk indicates degraded Parkin. (b) Alignment of the amino acid sequences surrounding Ser65 (marked by a black dot) from a variety of animal species. The numbers on the left correspond to the residue numbers of Parkin proteins. (c) Introduction of the S65A mutation delayed Parkin translocation to the depolarised mitochondria in PINK1 WT/GFP-Parkin/*PINK1*$^{-/-}$ MEFs. Cells retrovirally introduced with GFP-Parkin WT or its phospho-mutants (S65A and S65E) were treated with or without 30 μM CCCP for the indicated periods of time. GFP-Parkin and mitochondria were visualised with anti-GFP (green) and anti-Tom20 (red), respectively. Parkin signals are also shown as monochrome images. Scale bar = 10 μm. (d) Mitochondrial translocation efficiency of Parkin mutants. PINK1 WT/*PINK1*$^{-/-}$ MEFs stably expressing GFP-Parkin WT, S65A or S65E were treated as in (c). Cells expressing GFP-Parkin perfectly overlapped (Complete, examples are shown on the right), partially overlapped (Partial) or non-overlapped (No) with the Tom20 signal were counted. The data represent means ± SE from three experiments (n = 99–143 cells in each). ** $p < 0.01$, * $p < 0.05$ *vs.* WT at each time point.

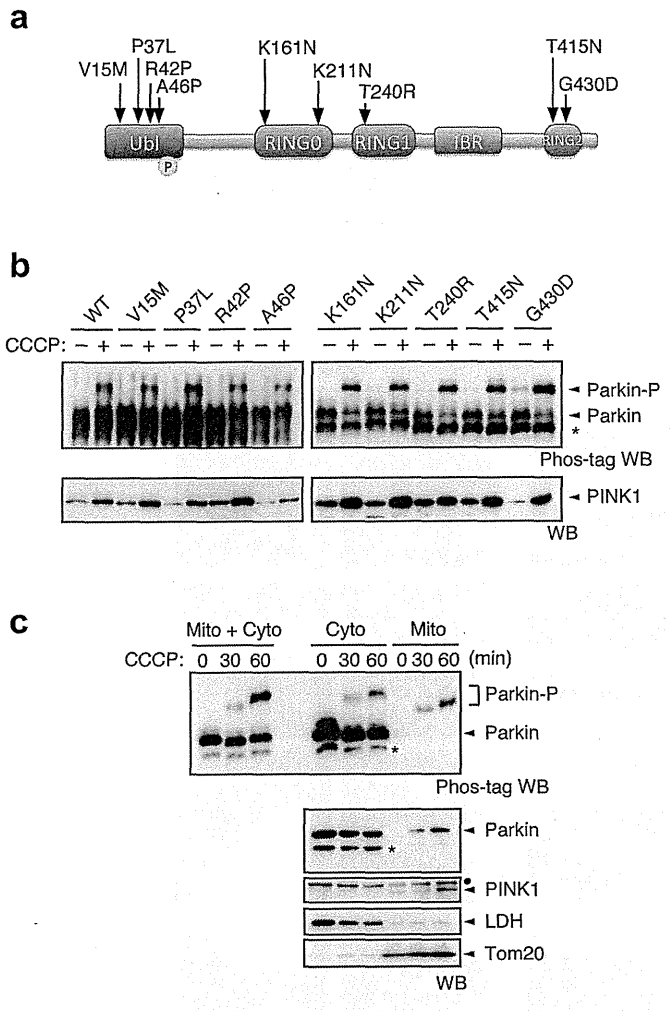

Figure 3 | Pathogenic mutants of Parkin are subjected to Ser65 phosphorylation. (a) Diagram of Parkin protein illustrating the pathogenic mutants used in this study. The Ser65 residue in the Ubl domain is shown as a yellow circle. RING, Ring-finger motif; IBR, in-between-Ring fingers domain. (b) Phos-tag Western blotting for Parkin and Western blotting for PINK1 were performed using Parkin WT and a series of pathogenic mutants as shown in Figure 2a. (c) Endogenous Parkin was also phosphorylated in SH-SY5Y cells after CCCP treatment. Post-nuclear cell lysates from SH-SY5Y cells treated with or without 10 μM CCCP for 30 and 60 min were fractionated into mitochondria-rich (Mito) and cytosolic (Cyto) fractions. These two fractions and their combination (Mito + Cyto) were subjected to Phos-tag or normal Western blotting analyses. Endogenous PINK1 was fractionated in the Mito fraction, as previously reported[45]. Lactate dehydrogenase (LDH) and Tom20 were used as cytosolic and mitochondrial marker proteins, respectively. Asterisks: putative cleaved Parkin; dots: non-specific bands.

accumulation of PINK1 (Fig. 4b). The impaired degradation cannot be explained simply by the delayed translocation of Parkin mutants because both mutants completed the mitochondrial translocation by the 6 hr time-point (data not shown and see Fig. 4c). In contrast, the profiles of Parkin expression and autoubiquitination in Parkin S65A- or S65E-expressing cells were comparable with those of WT (Fig. 4b). We also examined whether Ser65 mutations affect the accumulation of proteasome (Fig. 4c) and p62 (Supplementary Fig. S7) at the mitochondria during mitophagy via the immunostaining of the proteasome subunit alpha type 7 (α7) and p62. However, there was no evidence that Ser65 mutations inhibit or delay the recruitment of proteasome and p62 to the mitochondria. Finally, we tested whether the Parkin Ubl domain itself is indispensable for the mitochondrial translocation and the substrate degradation (Supplementary Fig. S8). Interestingly, Parkin mutant lacking the Ubl domain (ΔUbl) showed a mild delay in the mitochondrial translocation, slowed the mitochondrial reorganization to the perinuclear region (Supplementary Fig. S8b and c) and impaired the degradation of mitochondrial outer membrane proteins (Supplementary Fig. S8d). These results suggest that proper regulation of the Parkin Ubl domain through the Ser65 phosphorylation is required not only for efficient translocation to mitochondria as an initial step of mitophagy, but also for the degradation of mitochondrial outer membrane proteins during mitophagy through an as yet unknown mechanism.

Discussion

A series of *Drosophila* genetic and cell biological studies have clearly demonstrated that PINK1 is required for Parkin-mediated mitochondrial maintenance. The mitophagy of damaged mitochondria is a well-characterised event in which PINK1 and Parkin are involved. However, how PINK1 regulates Parkin is largely unclear. This study has shown that Ser65 in the Ubl domain of endogenous Parkin is phosphorylated in an activated PINK1-dependent manner. In addition to mitochondrial accumulation of PINK1, ΔΨm depolarisation-dependent PINK1 autophosphorylation has been reported to be an important element for PINK1 activation and Parkin recruitment[19,29]. Consistent with these observations, our investigation of PINK1 siRNA suggests that a lower level of PINK1 is able to phosphorylate Parkin afterΔΨm depolarisation (Fig. 1c, compare lanes 1 and 4). Our domain analysis of PINK1 demonstrates that intact PINK1 is required for CCCP-dependent Parkin phosphorylation, and the lack of phosphorylation in fibroblasts from a *PARK6* patient implies relevance to the pathogenesis of PD.

The biological significance of this phosphorylation event is suggested by the fact that replacement of Ser65 with alanine or glutamic acid impairs the mitochondrial translocation of Parkin and/or the subsequent mitophagy process. Our observation that maximal phosphorylation of Parkin occurs within 1 hr of CCCP treatment supports the idea that Ser65 phosphorylation is required for the early step of Parkin translocation. In contrast, PINK1 accumulation appears to last at least 6 hr (Fig. 4c and Supplementary Fig. S1b). The difference in time course between PINK1 accumulation and Parkin phosphorylation could be explained by the observation that phosphorylated Parkin is degraded by proteasomal activity. The biochemical evidence that only the phosphorylated form of endogenous Parkin is present in the mitochondrial fraction also implies that Parkin phosphorylation is an essential event for its mitochondrial translocation and subsequent activation (Fig. 3c and Supplementary Fig. S6). Overexpression of PINK1 and Parkin itself leads to mitochondrial translocation of Parkin independently of ΔΨm depolarization, which suggests that excessive amounts of PINK1 and Parkin do not faithfully reflect endogenous reactions. Our study using *PINK1*$^{-/-}$ MEFs stably co-expressing PINK1 and GFP-Parkin might also be saddled with such a problem. We believe that the endogenous observation in which phosphorylated Parkin is accumulated in mitochondria is a more reliable proposal as a molecular model. The delay of exogenous GFP-Parkin S65A in the mitochondrial translocation would indicate that modification of Ser65 is important for Parkin translocation at least. At the same time, another important finding is that pathogenic mutants that lose their translocation activity are also phosphorylated (Fig. 3b), raising the possibility that phosphorylation of Parkin at Ser65 is insufficient for translocation. Thus, Ser65 phosphorylation likely leads to other events in mitochondrial translocation, such as the association or dissociation of protein(s) involved in the mitochondrial translocation of Parkin or the modification of Parkin itself for activation at a different site(s).

Both the S65A and S65E Parkin mutants cannot undergo efficient mitophagy, as indicated by the incomplete degradation of

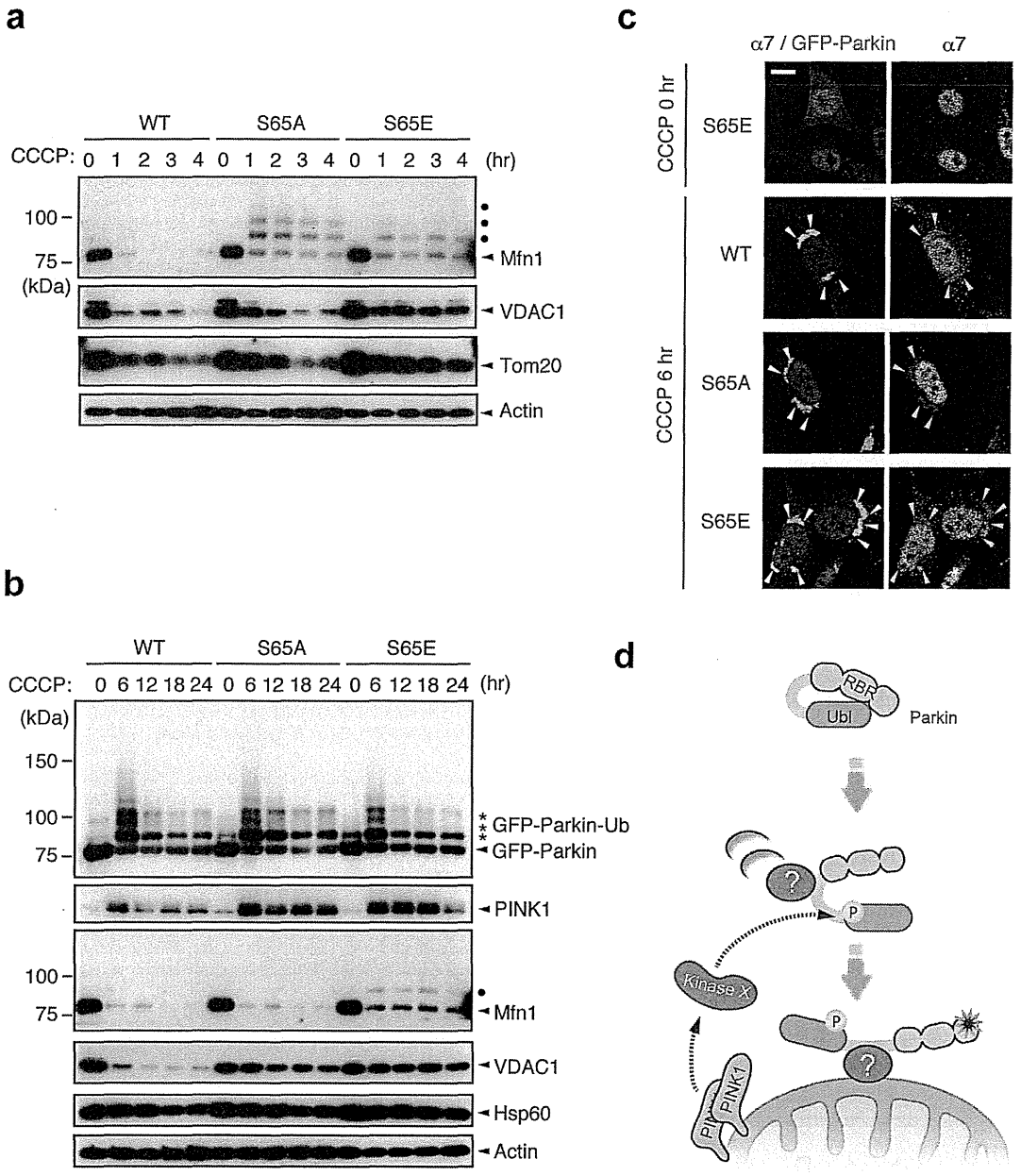

Figure 4 | Ser65 phosphorylation affects the subsequent autophagy reaction. (a) CCCP-dependent degradation of mitochondrial outer membrane proteins in PINK1 WT/*PINK1*$^{-/-}$ MEFs expressing WT or mutant forms of GFP-Parkin. Mfn1, VDAC1 and Tom20 were used as markers of mitochondrial outer membrane proteins. Actin: a loading control. Dots: ubiquitinated Mfn1. (b) Long-term time-course analysis of CCCP-dependent mitochondrial protein degradation. The degradation of outer membrane proteins was impaired in cells expressing GFP-Parkin S65A or S65E mutations. Hsp60 was used as a marker of mitochondrial matrix proteins. (c) S65A and S65E mutations do not affect proteasome recruitment to the mitochondria during mitophagy. PINK1 WT/*PINK1*$^{-/-}$ MEFs expressing WT or mutant forms of GFP-Parkin (green) were treated with 30 μM CCCP for 3 or 6 hr. Cells were stained with anti-proteasome subunit alpha type 7 (α7, red). α7-immunoreactivity was enriched in the nuclei of all three cell genotypes under normal conditions, as displayed in the representative image of S65E (CCCP 0 hr), and overlapped with the aggregated mitochondria (arrowheads) 6 hr after CCCP treatment irrespective of genotype. Similar results were obtained 3 hr after CCCP treatment. Scale bar = 10 μm. (d) Model for Parkin translocation and activation. The Parkin Ubl domain masks C-terminal RING-IBR-RING (RBR) domains for E3 activity[46]. A Parkin phosphorylation event at Ser65 (P), combined with unknown factor(s) (?), stimulates the mitochondrial translocation of Parkin, releasing the RBR domains from autoinhibition by the Ubl domain.

the mitochondrial outer membrane proteins. Because inhibition of the degradation of the mitochondrial outer membrane proteins by proteasome inhibitors is reported to block mitophagy[32,35], it may be that the modification of Parkin Ser65 has a greater than expected impact on the mitophagy process. Although our study does not demonstrate that the S65E mutant behaves exactly like the phosphorylated form of Parkin, the S65E mutant does translocate to the mitochondria in a similar way to WT, although with slightly impaired efficiency, suggesting that S65E has at least some properties that are similar to phosphorylated Parkin. Currently, it is unknown why S65E also inhibits the later processes of mitophagy. One possible explanation is that rapid degradation of phosphorylated Parkin is required for the proper progression of mitophagy, and S65E may not be degraded effectively. However,

there is no evidence that S65E is more stable than WT, as shown in Figure 4c.

Very recently, Kondapalli *et al.* proposed a model to explain the biological significance of Ser65 phosphorylation, in which Ser65 phosphorylation relieves autoinhibition of Parkin E3 activity by the Ubl domain[18]. This model may explain the depolarised $\Delta\Psi$m-dependent activation of Parkin. However, our data indicated that the Parkin S65A mutant is also autoubiquitinated (Fig. 4b) and that the ΔUbl mutant showed mild translocation defect and impaired substrate degradation (Supplementary Fig. S8). Moreover, if this is the case, the E3 activity of Parkin pathogenic mutants lacking mitochondrial translocational activity but harbouring intact E3 activity *in vitro* (such as K161N and K211N, which are subjected to the Ser65 phosphorylation) should be activated in the cytosol[38]. However, our previous data indicate that K161N and K211N are not activated by CCCP treatment[23]. Thus, it is conceivable that another step is required for depolarised $\Delta\Psi$m-dependent activation of Parkin E3. In addition, the Ubl domain might not only autoinhibit its E3 activity but also contribute to the mitochondrial translocation and the substrate degradation through an as yet unknown mechanism. We believe that an appropriate way to estimate Parkin E3 activity in the context of mitophagy is to evaluate the ubiquitination and degradation of substrates in cells with depolarised $\Delta\Psi$m. Mfn1 is a well-characterised direct substrate of Parkin[32], and Parkin-dependent poly-ubiquitination modification of Mfn1 can be detected by Western blotting upon $\Delta\Psi$m depolarisation[32,39,40]. Parkin S65A and S65E appear to ubiquitinate Mfn1, as poly-ubiquitinated forms of Mfn1 were observed (Fig. 4b). However, they cannot degrade it effectively, which suggests that the process of substrate degradation is also impaired in these mutants.

Kondapalli *et al.* have also shown that *T. castaneum* PINK1 (TcPINK1) directly phosphorylates human Parkin at Ser65[18]. We confirmed their finding using recombinant TcPINK1 produced from the same construct (Supplementary Fig. S4). The replacement of MBP-Parkin Ser65 with alanine completely abolished PINK1-mediated phosphorylation, indicating that Ser65 is the sole phosphorylation site *in vitro*. However, experiments in cultured cells showed that the replacement of Ser9, Ser10, Ser101 and Ser198 with alanine affects the Ser65 phosphorylation efficiency (Ser9, ~35% reduction; Ser10, ~76% reduction; Ser101, ~65% reduction; Ser198, ~92% reduction) (Fig. 2a). These residues might be priming phosphorylation sites for Ser65 phosphorylation.

Because PINK1 is believed to be activated in the mitochondria, a topological inconsistency arises from our cell-based data that cytosolic Parkin lacking the mitochondrial translocation activity is phosphorylated. Therefore, it is possible that PINK1 indirectly regulates Parkin phosphorylation. One possible explanation for this is the presence of another cytosolic kinase(s) regulated by PINK1 (Fig. 4d). Alternatively, because mitochondria are a dynamic organelle, cytosolic Parkin adjacent to the moving and fragmented mitochondria with depolarised $\Delta\Psi$m might be phosphorylated incidentally. The issue as to whether or not PINK1 directly phosphorylates Parkin in cells remains to be solved.

In conclusion, this study has suggested that PINK1-dependent Parkin phosphorylation at Ser65 accelerates the mitochondrial translocation of Parkin and showed that the introduction of mutations at this site also affects subsequent mitophagy processes. Concurrently, our data provide the possibility that there is an elaborate multi-step mechanism for the mitochondrial translocation of Parkin upon the loss of $\Delta\Psi$m (Fig. 4d), the clarification of which awaits further study.

Methods

Antibodies, plasmids and cell lines. Antibodies used in Western blot analysis were as follows: anti-Parkin (1 : 1,000 and 1 : 5,000 dilution for endogenous and exogenous Parkin, respectively; Cell Signaling Technology, clone PRK8), anti-PINK1 (1 : 1,000 dilution; Novus, BC100-494 or 1 : 1,000 dilution; Cell Signaling Technology, clone D8G3), anti-Mfn1 (1 : 1,000 dilution; Abnova, clone 3C9), anti-VDAC1 (1 : 1,000 dilution; Abcam, Ab15895), anti-Tom20 (1 : 500 dilution; Santa Cruz Biotechnology, FL-145), anti-FLAG-HRP (1 : 2,000 dilution; Sigma-Aldrich, clone M2), anti-GFP (1 : 5,000 dilution; Abcam, ab290), anti-Actin (1 : 10,000 dilution; Millipore, MAb1501), anti-LDH (1 : 1,000 dilution; Abcam, ab7639-1), anti-phospho-GSK3ß (1 : 1,000 dilution; Cell Signaling Technology, clone 5B3), anti-GSK3ß (1 : 1,000 dilution; Cell Signaling Technology, clone 27C10), and anti-Hsp60 (1 : 10,000 dilution; BD Biosciences, clone 24/Hsp60). Antibodies used in immunocytochemistry were as follows: FITC-conjugated anti-GFP (1 : 1,000 dilution; Abcam, ab6662), anti-Tom20 (1 : 1,000 dilution; Santa Cruz Biotechnology, FL-145), anti-Myc (1 : 500 dilution; Millipore, clone 4A6), anti-p62 (1: 500 dilution; Progen Biotechnik, GP62-C), anti-Parkin (1 : 1,000 dilution; Cell Signaling Technology, clone PRK8) and anti-proteasome α7 (1 : 250; a kind gift of Dr S. Murata at the University of Tokyo). cDNAs for human Parkin, PINK1 and its pathogenic and engineered mutants are as described in previous studies[23,41]. Parkin phospho-mutants were generated by PCR-based mutagenesis followed by sequencing confirmation of the entire gene. *PINK1*$^{-/-}$ MEFs, cultured as previously described[23], were retrovirally transfected with pMXs-puro harbouring non-tagged PINK1, PINK1-FLAG, non-tagged Parkin, HA-Parkin, GFP-Parkin and related cDNA; transfected cells were then selected with 1 μg/ml puromycin. HeLa cells maintained at 37°C in a 5% CO_2 atmosphere in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FCS and 1x non-essential amino acids (GIBCO) were retrovirally transfected with pMXs-puro harbouring non-tagged Parkin along with pcDNA3Hyg-mSlc7a1-VSVG and pcDNA3Hyg-mSlc7a1-FLAG (a kind gift of Dr N. Fujita at UCSD). Stable cell lines were selected with 1 μg/ml puromycin and cloned. Transient transfections of cultured cells were performed using Lipofectamine 2000 (Invitrogen) for plasmids and Lipofectamine RNAiMAX (Invitrogen) for stealth siRNA duplexes (Invitrogen), which were used according to the manufacturer's instructions.

Tissue culture. Skin biopsies were obtained from a *PARK6* case and a control without mutations in any known PD genes. The study was approved by the ethics committee of Juntendo University, and all participants gave written, informed consent. Dermal primary fibroblasts established from biopsies were cultured in high glucose DMEM supplemented with 10% foetal bovine serum, 1x non-essential amino acids, 1 mM sodium pyruvate (GIBCO), 100 μM 2-mercaptoethanol, and 1% penicillin–streptomycin at 37°C in a 5% CO_2 atmosphere.

Mapping of Parkin phosphorylation sites. *PINK1*$^{-/-}$ MEFs (6.0 x 10^7) expressing HA-Parkin and PINK1-FLAG were treated with or without 30 μM CCCP for 30 min. HA-Parkin (~500 ng in each) immunopurified with anti-HA-conjugated agarose beads was eluted with 8 M urea buffered with 50 mM Tris-HCl at pH 9.0. Samples from two independent experiments were digested with trypsin or chymotrypsin and analysed by nano-scale liquid chromatography-tandem mass spectrometry (Dionex Ultimate3000 RSLCnano and ABSciex TripleTOF 5600) followed by MASCOT searching and Mass Navigator/PhosPepAnalyzer processing for identification and label-free quantitation, respectively[42]. Determination of phosphosite localisation was performed based on the presence of site-determining ions[43].

Phosphorylation assay and mitochondrial fractionation. *PINK1*$^{-/-}$ MEFs harbouring HA-Parkin along with wild-type or a kinase-dead form of PINK1-FLAG were metabolically labelled with 175 μCi/ml of [^{32}P] orthophosphate in phosphate-free DMEM (GIBCO) with 10% FBS at 37°C for 3 hr. The medium was then replaced with fresh DMEM containing 10% FBS. Cells were treated with CCCP for 1.5 hr and were lysed on ice with lysis buffer containing 0.2% NP-40, 50 mM Tris (pH 7.4), 150 mM NaCl and 10% glycerol supplemented with protease inhibitor (Roche Diagnostics) and phosphatase inhibitor (Pierce) cocktails, and HA-Parkin and PINK1-FLAG were immunoprecipitated with anti-HA (Wako Pure Chemical, clone 4B2)- or anti-FLAG (Sigma-Aldrich, clone M2)-conjugated agarose beads. Immunoprecipitates were separated by SDS-PAGE and transferred onto a PVDF membrane. Autoradiography and Western blotting were performed to visualise proteins. Phos-tag Western blotting was performed as previously described[28]. Briefly, phospho-Parkin and phospho-PINK1 were separated on 8% gels containing 50 μM Phos-tag. Mitochondrial and cytosolic fractionations were performed as previously described, with some modifications[20]. The cytosolic fractions were further clarified by a second centrifugation at 105,000 *g* for 60 min to remove residual organelle membranes.

Immunocytochemical analysis. Cells plated on 3.5 mm glass-bottom dishes (MatTek) were fixed with 4% paraformaldehyde in PBS and permeabilised with 50 μg/ml digitonin for anti-Tom20 and anti-p62 staining or with 0.1% NP-40 for anti-α7 staining in PBS. Cells were stained with anti-Tom20 or anti-α7 antibodies in combination with FITC-conjugated anti-GFP antibody and were counterstained with DAPI for nuclei. Cells were imaged using laser-scanning microscope systems (TCS-SP5, Leica or LSM510 META, Carl Zeiss).

Statistical analysis. A one-way repeated measures ANOVA was used to determine significant differences between multiple groups unless otherwise indicated. If a significant result was achieved ($p < 0.05$), the means of the control and the specific test group were analysed using the Tukey-Kramer test.

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Author contributions

K.S., Y. Imai and N.H. designed the research; K.S., Y. Imai, S.Y., T.K. and Y. Ishihama performed the experiments; S.S. contributed new reagents/analytic tools; K.S. and Y. Imai analysed the data; and Y. Imai and N.H. wrote the paper. K.S. and Y. Imai contributed equally to this work.

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RESEARCH ARTICLE

VPS35 Mutation in Japanese Patients with Typical Parkinson's Disease

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ABSTRACT: Vacuolar protein sorting 35 *(VPS35)* was recently reported to be a pathogenic gene for late-onset autosomal dominant Parkinson's disease (PD), using exome sequencing. To date, *VPS35* mutations have been detected only in whites with PD. The aim of the present study was to determine the incidence and clinical features of Asian PD patients with *VPS35* mutations. We screened 7 reported nonsynonymous missense variants of *VPS35*, including p.D620N, known as potentially disease-associated variants of PD, in 300 Japanese index patients with autosomal dominant PD and 433 patients with sporadic PD (SPD) by direct sequencing or high-resolution melting (HRM) analysis. In addition, we screened 579 controls for the p.D620N mutation by HRM analysis. The p.D620N mutation was detected in 3 patients with autosomal dominant PD (1.0%), in 1 patient with SPD (0.23%), and in no controls. None of the other reported variants of *VPS35* were detected. Haplotype analysis suggested at least 3 independent founders for Japanese patients with p.D620N mutation. Patients with the *VPS35* mutation showed typical tremor-predominant PD. We report Asian PD patients with the *VPS35* mutation. Although *VPS35* mutations are uncommon in PD, the frequency of such mutation is relatively higher in Japanese than reported in other populations. In *VPS35*, p.D620N substitution may be a mutational hot spot across different ethnic populations. Based on the clinical features, *VPS35* should be analyzed in patients with PD, especially autosomal dominant PD or tremor-predominant PD.

Key Words: Parkinson's disease; *VPS35*; autosomal dominant; hotspot; mutation.

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