

ネフローゼにおける T 細胞分画の変化の検討は始まったばかりといえる。

MCNS 患者のリンパ球におけるエピゲノムの変化の検討も行われている。これまで MCNS におけるヒストンメチル化¹⁸⁾ や DNA のメチル化¹⁹⁾ の変化が報告されているが、現状ではこれが疾患発症の元となるリンパ球機能の変化と関連しているのかについてのデータはない。ステロイドはエピジェネティックな変化を誘導することから、MCNS におけるステロイド感受性のメカニズムを理解する上でこの分野は今後重要な領域となると予測される。

特発性ネフローゼ症候群と B 細胞機能の関わり

一般的な免疫応答の流れとしては、樹状細胞が異物を認識すると、まず T 細胞が活性化され、次に B 細胞が活性化される。活性化された B 細胞は、プラズマ細胞に分化し、抗体を産生する。現在 B 細胞は自己抗体産生に加え、その抗原提示能、共刺激分子発現能、サイトカイン産生能など多彩な機能を営むエフェクター B 細胞として機能していると考えられている。B 細胞は樹状細胞、T 細胞、マクロファージ・好中球などの免疫・炎症担当細胞との相互作用を通じて様々な病態へ深く関与している。

MCNS における B 細胞の機能については T 細胞に比べ研究が極めて乏しいが、臨床的には B 細胞抗原である CD20 に対するヒト型のモノクローナル抗体であるリツキシマブが頻回再発型ネフローゼ症候群に対して有効であること²⁰⁾、すなわち B 細胞を枯渇させることが MCNS の治療となることが明らかとなった。しかしこの効果がリツキシマブによる B 細胞に対する作用なのか、B 細胞を介した T 細胞機能の変化によるものかは明らかではない。一方でリツキシマブは acid sphingomyelinase-like phosphodiesterase 3b (SMPDL-3b) という糸球体上皮細胞に発現する蛋白と結合する。ネフローゼ患者血清は培養糸球体上皮細胞の SMPDL-3b の発現量を低下させ、その下流で細胞骨格の変化を惹起して濾過バリアー機能を低下させるが、リツキシマブは SMPDL-3b の発現量を上昇させ、患者血清による変化を抑制する²¹⁾。これはリツキシマブが免疫細胞を介さずに糸球体上皮細胞に直接作用して蛋白尿抑制作用を持つ可能性を示唆するものであるが、このメカニズムがリツキシマブの臨床的効果にどれほど関与しているのかは現時点では不明である。

その他の循環因子

MCNS と関連するその他の血中因子候補の一つとして Hemopexin が挙げられる。Hemopexin はヘムの代謝に関わる酵素で、ラットに Hemopexin を投与することにより可逆性の蛋白尿が惹起される²²⁾。Hemopexin 活性は MCNS 患者で増加しており²³⁾、Hemopexin は *in vitro* で Nephron を介して糸球体上皮細胞の細胞骨格に作用することから²⁴⁾、MCNS の一つのファクターである可能性がある。しかしこの報告は症例数が少なく、一般的な事象なのかどうかについては不明である。

もう一つの血中因子候補は Angiopoietin-like 4 (Angptl4) である。2011 年に Chugh らは MCNS 患者の血中に Angptl4 が増加していることを見出した。患者の糸球体においても Angptl4 の発現が上皮細胞で増強しており、マウスで糸球体上皮細胞特異的に Angptl4 を強発現させると蛋白尿を生ずることから、Angptl4 の上昇がネフローゼの原因の一つなのではないかと注目を集めた²⁵⁾。しかし、その可能性は現在では否定的と考えられる。それはその後の解析で肝臓において Angptl4 を発現させたマウスでは蛋白尿を呈しないことや、血中の Angptl4 は糸球体内皮細胞に働きかけ、むしろ蛋白尿を低下させる効果があること²⁶⁾ が判明したからである。興味深いことに Angptl4 濃度は血中アルブミンが低下することにより上昇するが、Angptl4 はリポ蛋白リパーゼ (LPL) の活性を抑制する作用を持つため、それによりトリグリセリドから FFA への変換が抑制され、高脂血症をきたす²⁶⁾。そのため Angptl4 はネフローゼにおける高脂血症の一端を担っている可能性が指摘されている。

ネフローゼ症候群の液性因子として 2011 年にマイアミ大学の Reiser のグループは FSGS 患者血清、特に再発時の血清で可溶性ウロキナーゼ受容体 (suPAR: soluble urokinase receptor) の濃度上昇がみられることを報告した²⁷⁾。suPAR はウロキナーゼ受容体から GPI-アンカー部が切断されて可溶性となる血中循環蛋白である²⁸⁾。大規模なコホートにおいても巣状糸球体硬化症患者で血清中の suPAR 濃度の上昇が確認され²⁹⁾、またこの蛋白をマウスに投与することにより蛋白尿が生じることなどから、suPAR が糸球体硬化症の原因因子の一つ、さらには FSGS と MCNS の病態の違いを説明する因子ではないかと考えられた。しかし多数の別のグループによる検討により suPAR は腎機能低下をきたす他の疾患でも上昇し、suPAR 濃度は糸球体濾過量と負の相関を示すことが判明し、腎機能との相関以上の suPAR 濃度上昇の意

義はないとする報告が相次いでいる³⁰⁾⁻³³⁾。現状ではネフローゼの病因としての suPAR については否定的と考えざるを得ない。

CD80 (B7-1)

一般的な抗原提示において、異物は樹状細胞やマクロファージなどの抗原提示細胞によって取り込まれ抗原として提示される。T 細胞は T 細胞受容体 (T cell receptor: TCR) を介して抗原提示細胞上の抗原情報を認識し、自ら活性化して分裂したりエフェクター細胞へと分化する。この情報交換の際、T 細胞は抗原提示細胞と強固に接着し、その接着面に“免疫シナプス”を形成する。CD80 (B7-1) は膜蛋白質であり、活性化 B 細胞と抗原提示細胞に発現する。CD80 は TCR の活性化に呼応して CD4+T 細胞上の CD28 と結合し、T 細胞の増殖を促進する。このように CD80 と CD28 の間の相互作用 / 共刺激シグナル伝達は T 細胞と B 細胞または抗原提示細胞間での重要な情報伝達を担っており、獲得免疫応答を調整している。一方、負の補助刺激受容体である CTLA-4 も CD80 をリガンドとして結合するが、その親和性が CD28 と CD80 の結合の数十倍と高いため CD28 と CD80 の結合、すなわち CD28 シグナルを強力に阻害する。

糸球体上皮細胞が刺激を受け傷害されると CD80 を発現するようになることが動物実験により明らかにされた³⁴⁾。また MCNS の再発時に尿中の CD80 濃度が上昇するが、これは寛解期や FSGS 患者ではみられないことから、CD80 の発現変化は MCNS で特異的にみられる現象である可能性も示唆されていた³⁵⁾。MCNS 患者の血清を培養ポドサイトに添加すると CD80 の発現が上昇することが *in vitro* でも示されており³⁶⁾、MCNS と CD80 発現量との間には密接な関連があると考えられる。このようにまず血清中の何らかの刺激による CD80 の発現誘導を 1st hit、その後の CD80 のシグナルを抑制する CTLA4 の発現低下を 2nd hit とし、それらの結果 CD80 の恒常的に活性が上昇することが MCNS の原因であるとする two-hit 仮説も提唱され始めている³⁷⁾。

アバタセプトは CTLA4 と IgG のキメラタンパク質であり、CD80 と結合することにより CD80-CD28 シグナルを抑制し免疫応答を減弱させる。そこでアバタセプトにより糸球体上皮細胞上の CD80 を抑制することが蛋白尿の減弱につながるのか、最近複数のグループにより検討が行われた。Yu らは 5 人の FSGS 患者 (4 人はリツキシマブ抵抗性、1 人はステロイド抵抗性ネフローゼ症候

群) にアバタセプトを投与し、いずれの患者においてもネフローゼレベルの蛋白尿が改善したと報告した³⁸⁾。一方で、Garin らはアバタセプトが MCNS 患者において一時的な蛋白尿抑制効果を持ったのに対して、FSGS 患者では尿中 CD80 抗原が減少するにもかかわらず、蛋白尿に変化がなかったと報告している³⁹⁾。また別のグループからアバタセプトが FSGS 患者の蛋白尿への効果は乏しいことが報告されている⁴⁰⁾。ネフローゼ症候群における CD80 の作用、およびアバタセプトの効果については今後の症例の集積が必要である。

ポドサイト因子

先天性ネフローゼ症候群やステロイド抵抗性ネフローゼ症候群では原因遺伝子が明らかになりつつある (表 2)。これらは発症年齢により、先天性ネフローゼ症候群として生後早期に症状の出るもの (*NPHS1*, *NPHS2*, *NPHS3*, *CD2AP*, *MYO1E*, *PTPRO*)、常染色体優性遺伝の形式をとる成人発症のもの (*TRPC6*, *ACTN4*, *INF2*)、他臓器症状を伴うもの (*WT1*, *LAMB2*, *LMX1B*, *MYH9*)、と便宜上 3 つに大きく分類することができる。これらの遺伝子産物のうち *LAMB2* は基底膜成分である Laminin $\beta 2$ をコードするが、その他の多くの遺伝子は糸球体上皮細胞に強く発現する蛋白質をコードしているため、これらの疾患はポドサイト病 (Podocytopathy) の一病態ととらえることができる。なお、欧米の検討では生後 1 年以内に発症する乳児ネフローゼ症候群の 3 分の 2 は 4 つの遺伝子変異で説明され (*NPHS1*-24%, *NPHS2*-38%, *LAMB2*-5%, *WT1*-3%), 2 歳未満発症のステロイド抵抗性ネフローゼ症候群 / 先天性ネフローゼ症候群においては現在判明しているうちの 24 遺伝子の変異が 9 割近くに見出されることも報告されている⁴¹⁾。ステロイド抵抗性ネフローゼ症候群においても 2000 名を超える症例の解析から既知の 27 遺伝子で説明される症例が 3 割に及ぶことが報告されている⁴²⁾。

ステロイド感受性ネフローゼ症候群 (SSNS) や MCNS では遺伝的な背景がどの程度発症に関与しているのだろうか? ステロイド感受性ネフローゼ症候群の同胞内での発症は 3% とされており、家族的な発症自体は稀である⁴³⁾。確かに SRNS に比べて SSNS では既知の遺伝子異常の頻度は極めて低く、例えば 38 名の SSNS の解析では遺伝子異常を有するものは見つからなかった⁴⁴⁾。しかし病理学的に MCNS とされている症例においても遺伝子変異が原因となる症例も存在する。例えば本邦の蛋白尿を繰り返す兄弟において軽微な Nephrin の異常が

表2 ネフローゼ症候群の遺伝因子

	遺伝子	遺伝子座	遺伝形式	OMIM	腎組織	蛋白	役割	
1. 主に小児期に発症するもの	<i>NPHS1</i>	19q13.1	AR	602716	FSGS/MGC	Nephrin	スリット膜成分	
	<i>NPHS2</i>	1q25-31	AR	604766	FSGS/MGC	Podocin	スリット膜成分	
	<i>NPHS3</i>	10q23-24	AR	610725	DMS/FSGS	PLCe1	?	
	<i>WT1</i>	11p13	AD(女性)	256370	DMS/FSGS	WT1	転写因子	
	<i>CD2AP</i>	6p12.3	AR	607832	FSGS	CD2AP	スリット膜成分	
	<i>PTPRO</i>	12p12	AR	600579	FSGS/MGC	GLEPP1	頂端面膜蛋白	
	<i>MYO1E</i>	15q22.2	AR	601479	FSGS	Myosin1e	足突起—細胞骨格	
	<i>ARHGDLA</i>	17q25.3	AR	601925	DMS/FSGS	RhoGDI	細胞骨格	
	<i>ADCK4</i>	19q13.2	AR	615567	FSGS	ADCK4	酵素	
	<i>EMP2</i>	16p13.13	AR	602334	MCD	EMP2	膜蛋白	
	<i>CRB2</i>	9q33.3	AR	609720	FSGS	Crumbs2	膜蛋白	
	<i>COQ2</i>	4q21-22	AR	609825	FSGS/CG	COQ2	ミトコンドリア酵素	
	<i>LMX1B</i>	9q34.1	AD	161200	FSGS/MCNS	LMX1b	転写因子	
2. 主に成人発症するもの	<i>INF2</i>	14q32.33	AD	613237	FSGS	INF2	足突起—細胞骨格	
	<i>ACTN4</i>	19q13	AD	604638	FSGS	α -actinin4	足突起—細胞骨格	
	<i>ARHGAP24</i>	4q21	AD	610586	FSGS	Filgap	Rho 蛋白質	
	<i>ANLN</i>	7p14.2	AD	616027	FSGS	Anilin	足突起—細胞骨格	
	<i>TRPC6</i>	11q21-22	AD	603652	FSGS	TRPC6	スリット膜成分	
3. 他臓器症状を伴うもの	眼異常	<i>LAMB2</i>	3p21	AR	609049	DMS/FSGS	Laminin β 2	基底膜成分
	性分化異常, Wilms 腫瘍	<i>WT1</i>	11p13	AD	194080	DMS	WT1	転写因子
	性分化異常	<i>WT1</i>	11p13	AD	136680	FSGS	WT1	転写因子
	爪, 骨変形	<i>LMX1B</i>	9q34.1	AD	161200	FSGS/MCNS	LMX1b	転写因子
	脳室拡大	<i>CRB2</i>	9q33.3	AR	609720	FSGS	Crumbs2	膜蛋白
	骨形成不全, 免疫不全	<i>SMARCAL1</i>	2q35	AR	606622	FSGS	SMARCAL1	細胞骨格
	神経症状	<i>COQ2</i>	4q21-22	AR	609825	FSGS/CG	COQ2	ミトコンドリア酵素
	神経症状, 難聴	<i>COQ6</i>	14q24.3	AR	614647	FSGS	COQ6	ミトコンドリア酵素
	神経症状	<i>PDSS2</i>	6q21	AR	610564	FSGS	DLP1	ミトコンドリア酵素
	神経症状	<i>MTIL1</i>	ミトコンドリア		590050	FSGS	MTIL1	ミトコンドリア酵素
	神経症状	<i>SCARB2</i>	4q21.1	AR	602257	FSGS	LIMP2	リソソーム
	表皮水疱症, 幽門閉鎖	<i>ITGB4</i>	17q25.1	AR	147557	FSGS	Integrin β 4	基底膜/細胞接着
	表皮水疱症, 難聴	<i>CDI51</i>	11p15.1	AR	602243	基底膜異常	Tetraspanin	基底膜/細胞接着
	早老症	<i>ZMPSTE24</i>	1p34	AR	606480	FSGS	STE24	?
	間質性肺疾患, 表皮水疱症	<i>ITGA3</i>	17q21.33	AR	605025	FSGS	Integrin α 3	基底膜/細胞接着
	血小板異常	<i>MYH9</i>	22q11.2	AD	160775	FSGS	Myosin Heavy Chain 9	足突起—細胞骨格
4. 疾患関連遺伝子	<i>GPC5</i>	13q31.3		602446	NS	GPC5	?	
	<i>HLA-DQA1</i>	6p21.32		146880	SSNS	HLA-DQA1	T 細胞シグナル	
	<i>PLCG2</i>	16q23.3		600220	SSNS	Phospholipase C γ 2	Ca シグナル	
	<i>APOL1</i>	22q12.3		603743	FSGS	Apolipoprotein L-1	?	
	<i>MYO1E</i>	15q22.2		601479	FSGS	Myosin1e	足突起—細胞骨格	
	<i>CUBN</i>	10p13		602997		Cubilin	受容体	

AR: 常染色体劣性遺伝, AD: 常染色体優性遺伝

DMS: びまん性メサンギウム硬化症, FSGS: 巣状分節性糸球体硬化症, CG: 虚脱性糸球体腎症, MGC: 微小糸球体病変

報告されている⁴⁵⁾。また Nail-Patella 症候群の原因遺伝子 *LMX1B* の変異が腎外症状を呈さずに蛋白尿のみを呈する患者において見出されている⁴⁶⁾。さらに、幼少期発症の家族性 SSNS の解析により *EMP2* の遺伝子変異が見出されている⁴⁷⁾。*EMP2* は糸球体上皮細胞や内皮細胞に発現しており、膜蛋白質のカベオリンの発現を制御しており、その変異が上皮細胞の形態変化の原因となると考えられる。またその他の遺伝子異常として、SRNS の原因として見出された kidney ankyrin repeat-containing pro-

tein 1 (KANK) 1, 2, 4 の変異が SSNS/MCNS 患者にも見出されている⁴⁸⁾。これらの事実から、糸球体上皮細胞機能に影響する遺伝子変異は必ずしも先天性ネフローゼ症候群や SRNS という病像を呈さずに、SSNS/MCNS の原因ともなりうる事が示唆される。

ある遺伝子変異の有無によって病気の発症が決定されるような原因遺伝子のみではなく、感受性遺伝子と呼ばれるような、発症のリスクをあげる遺伝子多型についても大規模な検討が始まっている。複数の感受性遺伝子を

原因とする疾患の場合、それぞれの遺伝子をもつ発症リスクの大きさは「オッズ比」であらわされる。具体的には、感受性遺伝子をもたない人に比べて、感受性遺伝子をもつ人のリスクが何倍になるかという数値で表される。ゲノムワイド関連解析(Genome Wide Association Study: GWAS)は、ヒトの全ゲノム中に1000万種以上もあるといわれるSNPs(一塩基多型:ゲノム上で一塩基だけが他のものに置き換わっている変異のうち、特定の集団の1%以上にみられるものをいう)の代表的なものをマーカーとして使い、特定の個人が全ゲノム中にどのようなSNPsをもつのかを網羅的に検討するものである。本邦の200例弱の後天性ネフローゼ症候群を対象としたGWASが行われ、Glypican-5をコードするGPC5のイントロンにおけるSNP多型がネフローゼ症候群の発症と相関することが報告された。Glypican-5は糸球体上皮細胞に発現しているが、糸球体上皮細胞特異的にGlypican-5をノックダウンしたマウスでは実験的な蛋白尿発症に抵抗性を示すことから、この遺伝子の発現量により糸球体上皮の傷害への感受性が規定されると考えられている⁴⁹⁾。また、200例ほどの小児期発症ステロイド感受性ネフローゼ症候群のGWAS解析では、6番染色体上のHLA-DQA1の多型の割合がSSNSで有意に上昇していた(オッズ比2.1)⁵⁰⁾。これらの多型そのものだけでは疾患の発症する訳ではないが、こういった複数の感受性遺伝子の関与がネフローゼ発症のリスクに大きく影響する可能性がある。今後広まると考えられるこれらの巨視的ゲノム解析は感受性遺伝子の動態を明らかにするのみならず、特定の民族や人種などの集団にみられる遺伝的な構造を明らかにするためにも有効である。

なぜ未だにMCNSの原因がわからないのか？

ネフローゼ症候群については古くは15世紀から記載があり、これまで述べた通り1970年代からその原因について様々な検討が行われてきた。先天性ネフローゼ症候群やステロイド抵抗性ネフローゼ症候群については遺伝子異常が明らかになり、多くの部分が糸球体上皮細胞異常で説明されることが判明しているが、MCNSについては現在に至ってもまだ不明な部分が多い。この理由には以下のような様々な可能性が考えられる。

- (1) 現在解析されている血中因子(サイトカイン等を含む)以外の因子による
- (2) 原因(遺伝的、免疫的背景あるいは循環因子)が単一ではない
- (3) 複数の因子の組み合わせによって発症する(例:糸球

体上皮細胞因子+免疫因子, T細胞因子+B細胞因子, 1st hit+2nd hit など)

- (4) 遺伝的な複数の因子の複合した結果発症する(Double Heterozygosity など)

また、ネフローゼ症候群の原因解析に関する研究の困難な理由も様々挙げられる。

- (1) 信頼できる in vitro 再現系, 動物モデルが確立されていない
- (2) ステロイド等の免疫抑制薬の投与が行われるため, 治療後の血液あるいは尿サンプルを解析に含めるとその影響を無視できない
- (3) 小児特有の疾患であり, FSGSを含めた内科領域の研究に比べて研究者および研究費が少ない
- (4) 原因究明を目的とした大規模なレジストリーが行われていない

今後の検討としてはこれらの問題点を踏まえ、ゲノム/エピゲノム/プロテオーム/トランスクリプトームなどの網羅的解析を大規模なコホートを用いて行う必要があると考えられる。また家族歴のある患者の遺伝的背景を明らかにすることが、より一般的な特発性ネフローゼ症候群の発症原因に迫る契機となるかもしれない。

結 び

特発性ネフローゼ症候群の治療の中心は依然としてステロイドを基礎とした免疫抑制療法であり、多彩な副作用が問題となる。ネフローゼ症候群の原因解明の最終的な目標は原因を元にした特異的な治療や予防法を見出すことであり、今後の検討がステロイドに代わる新たな創薬など革新的治療法の開発につながることを期待される。

「日本小児腎臓病学会の定める基準に基づく利益相反に関する開示事項はありません。」

文 献

- 1) Shalhoub RJ: Pathogenesis of lipid nephrosis: a disorder of T-cell function. *Lancet* 1974; 2: 556-560.
- 2) Lagrue G, Xheneumont S, Branellac A, Hirbec G, Weil B: A vascular permeability factor elaborated from lymphocytes. I. Demonstration in patients with nephrotic syndrome. *Biomedicine* 1975; 23: 37-40.
- 3) Koyama A, Fujisaki M, Kobayashi M, Igarashi M, Narita M: A glomerular permeability factor produced by human T cell hybridomas. *Kidney Int* 1991; 40: 453-460.
- 4) Pereira Wde F, Brito-Melo GE, Guimaraes FT, Carvalho TG, et al: The role of the immune system in idiopathic nephrotic syndrome. a

- review of clinical and experimental studies. *Inflamm Res* 2014; 63: 1-12.
- 5) Araya CE, Wasserfall CH, Brusko TM, Mu W, Segal MS, Johnson RJ, Garin EH: A case of unfulfilled expectations. Cytokines in idiopathic minimal lesion nephrotic syndrome. *Pediatr Nephrol* 2006; 21: 603-610.
 - 6) Kanai T, Shiraishi H, Yamagata T, Ito T, Odaka J, Saito T, Aoyagi J, Momoi MY: Th2 cells predominate in idiopathic steroid-sensitive nephrotic syndrome. *Clin Exp Nephrol* 2010; 14: 578-583.
 - 7) Yap HK, Cheung W, Murugasu B, Sim SK, Seah CC, Jordan SC: Th1 and Th2 cytokine mRNA profiles in childhood nephrotic syndrome: evidence for increased IL-13 mRNA expression in relapse. *J Am Soc Nephrol* 1999; 10: 529-537.
 - 8) Mishra OP, Teli AS, Singh U, Abhinay A, Prasad R: Serum immunoglobulin E and interleukin-13 levels in children with idiopathic nephrotic syndrome. *J Trop Pediatr* 2014; 60: 467-471.
 - 9) Komatsuda A, Wakui H, Iwamoto K, Togashi M, Masai R, Maki N, Sawada K: GATA-3 is upregulated in peripheral blood mononuclear cells from patients with minimal change nephrotic syndrome. *Clin Nephrol* 2009; 71: 608-616.
 - 10) Van Den Berg JG, Aten J, Chand MA, Claessen N, Dijkink L, Wijdenes J, Lakkis FG, Weening JJ: Interleukin-4 and interleukin-13 act on glomerular visceral epithelial cells. *J Am Soc Nephrol* 2000; 11: 413-422.
 - 11) Lai KW, Wei CL, Tan LK, Tan PH, Chiang GS, Lee CG, Jordan SC, Yap HK: Overexpression of interleukin-13 induces minimal-change-like nephropathy in rats. *J Am Soc Nephrol* 2007; 18: 1476-1485.
 - 12) Grimbert P, Valanciute A, Audard V, Pawlak A, Le gouvelo S, Lang P, Niaudet P, Bensman A, Guellaen G, Sahali D: Truncation of C-mip (Tc-mip), a new proximal signaling protein, induces c-maf Th2 transcription factor and cytoskeleton reorganization. *J Exp Med* 2003; 198: 797-807.
 - 13) Zhang SY, Kamal M, Dahan K, Pawlak A, Ory V, Desvaux D, Audard V, Candelier M, BenMohamed F, Mohamed FB, Matignon M, Christov C, Decrouy X, Bernard V, Mangiapan G, Lang P, Guellaen G, Ronco P, Sahali D: c-mip impairs podocyte proximal signaling and induces heavy proteinuria. *Sci Signal* 2010; 3: ra39.
 - 14) Bitzan M, Babayeva S, Vasudevan A, Goodyer P, Torban E: TNF α pathway blockade ameliorates toxic effects of FSGS plasma on podocyte cytoskeleton and β 3 integrin activation. *Pediatr Nephrol* 2012; 27: 2217-2226.
 - 15) Sahali D, Pawlak A, Le Gouvello S, Lang P, Valanciute A, Remy P, Loirat C, Niaudet P, Bensman A, Guellaen G: Transcriptional and post-transcriptional alterations of IkappaBalpha in active minimal-change nephrotic syndrome. *J Am Soc Nephrol* 2001; 12: 1648-1658.
 - 16) Araya C, Diaz L, Wasserfall C, Atkinson M, Mu W, Johnson R, Garin E: T regulatory cell function in idiopathic minimal lesion nephrotic syndrome. *Pediatr Nephrol* 2009; 24: 1691-1698.
 - 17) Hashimura Y, Nozu K, Kanegane H, Miyawaki T, Hayakawa A, Yoshikawa N, Nakanishi K, Takemoto M, Iijima K, Matsuo M: Minimal change nephrotic syndrome associated with immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome. *Pediatr Nephrol* 2009; 24: 1181-1186.
 - 18) Zhang L, Dai Y, Peng W, Lu J, Zhang Y, Wang L: Genome-wide analysis of histone H3 lysine 4 trimethylation in peripheral blood mononuclear cells of minimal change nephrotic syndrome patients. *Am J Nephrol* 2009; 30: 505-513.
 - 19) Kobayashi Y, Aizawa A, Takizawa T, Yoshizawa C, Horiguchi H, Ikeuchi Y, Kakegawa S, Watanabe T, Maruyama K, Morikawa A, Hatada I, Arakawa H: DNA methylation changes between relapse and remission of minimal change nephrotic syndrome. *Pediatr Nephrol* 2012; 27: 2233-2241.
 - 20) Iijima K, Sako M, Nozu K, Mori R, Tsuchida N, Kamei K, Miura K, Aya K, Nakanishi K, Ohtomo Y, Takahashi S, Tanaka R, Kaito H, Nakamura H, Ishikura K, Ito S, Ohashi Y: Rituximab for Childhood-onset Refractory Nephrotic Syndrome (RCRNS) Study Group: Rituximab for childhood-onset, complicated, frequently relapsing nephrotic syndrome or steroid-dependent nephrotic syndrome: a multicentre, double-blind, randomised, placebo-controlled trial. *Lancet* 2014; 384: 1273-1281.
 - 21) Fomoni A, Sageshima J, Wei C, Merscher-Gomez S, Aguillon-Prada R, Jauregui AN, Li J, Mattiazzi A, Ciancio G, Chen L, Zilleruelo G, Abitbol C, Chandar J, Seehunvong W, Ricordi C, Ikehata M, Rastaldi MP, Reiser J, Burke GW 3rd.: Rituximab targets podocytes in recurrent focal segmental glomerulosclerosis. *Sci Transl Med* 2011; 3: 85ra46.
 - 22) Cheung PK, Klok PA, Baller JF, Bakker WW: Induction of experimental proteinuria in vivo following infusion of human plasma hemopexin. *Kidney Int* 2000; 57: 1512-1520.
 - 23) Bakker WW, van Dael CM, Pierik LJ, van Wijk JA, Nauta J, Borghuis T, Kapoos JJ: Altered activity of plasma hemopexin in patients with minimal change disease in relapse. *Pediatr Nephrol* 2005; 20: 1410-1415.
 - 24) Lennon R, Singh A, Welsh GI, Coward RJ, Satchell S, Ni L, Mathieson PW, Bakker WW, Saleem MA: Hemopexin induces nephrin-dependent reorganization of the actin cytoskeleton in podocytes. *J Am Soc Nephrol* 2008; 19: 2140-2149.
 - 25) Clement LC, Avila-Casado C, Macé C, Soria E, Bakker WW, Kersten S, Chugh SS: Podocyte-secreted angiopoietin-like-4 mediates proteinuria in glucocorticoid-sensitive nephrotic syndrome. *Nat Med* 2011; 17: 117-122.
 - 26) Clement LC, Macé C, Avila-Casado C, Joles JA, Kersten S, Chugh SS: Circulating angiopoietin-like 4 links proteinuria with hypertriglyceridemia in nephrotic syndrome. *Nat Med* 2014; 20: 37-46.
 - 27) Wei C, El Hindi S, Li J, Fomoni A, Goes N, Sageshima J, Maiguel D, Karumanchi SA, Yap HK, Saleem M, Zhang Q, Nikolic B, Chaudhuri A, Daftarian P, Salido E, Torres A, Salifu M, Sarwal MM, Schaefer F, Morath C, Schwenger V, Zeier M, Gupta V, Roth D, Rastaldi MP, Burke G, Ruiz P, Reiser J: Circulating urokinase receptor as a cause of focal segmental glomerulosclerosis. *Nat Med* 2011; 17: 952-960.
 - 28) Sidenius N, Sier CF, Blasi F: Shedding and cleavage of the urokinase receptor (uPAR): identification and characterisation of uPAR fragments in vitro and in vivo. *FEBS Lett* 2000; 475: 52-56.
 - 29) Wei C, Trachtman H, Li J, Dong C, Friedman AL, Gassman JJ, McMahan JL, Radeva M, Heil KM, Trautmann A, Anarat A, Emre S, Ghiggeri GM, Ozaltin F, Haffner D, Gipson DS, Kaskel F, Fischer DC, Schaefer F, Reiser J: PodoNet and FSGS CT Study Consortia: Circulating suPAR in two cohorts of primary FSGS. *J Am Soc Nephrol* 2012; 23: 2051-2059.
 - 30) Meijers B, Maas RJ, Sprangers B, Claes K, Poesen R, Bammens B, Naesens M, Deegens JK, Dietrich R, Storr M, Wetzels JF, Evenepoel P, Kuypers D: The soluble urokinase receptor is not a clinical marker for focal segmental glomerulosclerosis. *Kidney Int* 2014; 85: 636-640.
 - 31) Bock ME, Price HE, Gallon L, Langman CB: Serum soluble urokinase-type plasminogen activator receptor levels and idiopathic

- FSGS in children: a single-center report. *Clin J Am Soc Nephrol* 2013; 8: 1304-1311.
- 32) Harita Y, Ishizuka K, Tanego A, Sugawara N, Chikamoto H, Akioka Y, Tsurumi H, Miura K, Gotoh Y, Tsujita M, Yamamoto T, Horike K, Takeda A, Oka A, Igarashi T, Hattori M: Decreased glomerular filtration as the primary factor of elevated circulating suPAR levels in focal segmental glomerulosclerosis. *Pediatr Nephrol* 2014; 29: 1553-1560.
 - 33) Wada T, Nangaku M, Maruyama S, Imai E, Shoji K, Kato S, Endo T, Muso E, Kamata K, Yokoyama H, Fujimoto K, Obata Y, Nishino T, Kato H, Uchida S, Sasatomi Y, Saito T, Matsuo S: A multicenter cross-sectional study of circulating soluble urokinase receptor in Japanese patients with glomerular disease. *Kidney Int* 2014; 85: 641-648.
 - 34) Reiser J, von Gersdorff G, Loos M, Oh J, Asanuma K, Giardino L, Rastaldi MP, Calvaresi N, Watanabe H, Schwarz K, Faul C, Kretzler M, Davidson A, Sugimoto H, Kalluri R, Sharpe AH, Kreidberg JA, Mundel P: Induction of B7-1 in podocytes is associated with nephrotic syndrome. *J Clin Invest* 2004; 113: 1390-1397.
 - 35) Garin EH, Mu W, Arthur JM, Rivard CJ, Araya CE, Shimada M, Johnson RJ: Urinary CD80 is elevated in minimal change disease but not in focal segmental glomerulosclerosis. *Kidney Int* 2010; 78: 296-302.
 - 36) Ishimoto T, Cara-Fuentes G, Wang H, Shimada M, Wasserfall CH, Winter WE, Rivard CJ, Araya CE, Saleem MA, Mathieson PW, Johnson RJ, Garin EH: Serum from minimal change patients in relapse increases CD80 expression in cultured podocytes. *Pediatr Nephrol* 2013; 28: 1803-1812.
 - 37) Ishimoto T, Shimada M, Araya CE, Huskey J, Garin EH, Johnson RJ: Minimal change disease: a CD80 podocytopathy? *Semin Nephrol* 2011; 31: 320-325.
 - 38) Yu CC, Fornoni A, Weins A, Hakroush S, Maiguel D, Sageshima J, Chen L, Ciancio G, Faridi MH, Behr D, Campbell KN, Chang JM, Chen HC, Oh J, Faul C, Arnaout MA, Fiorina P, Gupta V, Greka A, Burke GW, Mundel P: Abatacept in B7-1-positive proteinuric kidney disease. *N Engl J Med* 2013; 369: 2416-2423.
 - 39) Garin EH, Reiser J, Cara-Fuentes G, Wei C, Matar D, Wang H, Alachkar N, Johnson RJ: Case series: CTLA4-IgG1 therapy in minimal change disease and focal segmental glomerulosclerosis. *Pediatr Nephrol* 2015; 30: 469-477.
 - 40) Alkandari O, Nampoory N, Nair P, Atta A, Zakaria Z, Mossad A, Yagan J, Al-Otaibi T: Recurrent focal segmental glomerulosclerosis and abatacept. *Case Report. Exp Clin Transplant* 2015.
 - 41) Rood IM, Deegens JK, Wetzels JF: Genetic causes of focal segmental glomerulosclerosis: implications for clinical practice. *Nephrol Dial Transplant* 2012; 27: 882-890.
 - 42) Sadowski CE, Lovric S, Ashraf S, Pabst WL, Gee HY, Kohl S, Engelmann S, Vega-Warner V, Fang H, Halbritter J, Somers MJ, Tan W, Shril S, Fessi I, Lifton RP, Bockenbauer D, El-Desoky S, Kari JA, Zenker M, Kemper MJ, Mueller D, Fathy HM, Soliman NA; SRNS Study Group, Hildebrandt F: A single-gene cause in 29.5% of cases of steroid-resistant nephrotic syndrome. *J Am Soc Nephrol* 2015; 26: 1279-1289.
 - 43) Xia Y, Mao J, Jin X, Wang W, Du L, Liu A: Familial steroid-sensitive idiopathic nephrotic syndrome: seven cases from three families in China. *Clinics (Sao Paulo)* 2013; 68: 628-631.
 - 44) Giglio S, Provenzano A, Mazzinghi B, Becherucci F, Giunti L, Sansavini G, Ravaglia F, Roperto RM, Farsetti S, Benetti E, Rotondi M, Murer L, Lazzeri E, Lasagni L, Materassi M, Romagnani P: Heterogeneous genetic alterations in sporadic nephrotic syndrome associate with resistance to immunosuppression. *J Am Soc Nephrol* 2015; 26: 230-236.
 - 45) Kitamura A, Tsukaguchi H, Hiramoto R, Shono A, Doi T, Kagami S, Iijima K: A familial childhood-onset relapsing nephrotic syndrome. *Kidney Int* 2007; 71: 946-951.
 - 46) Isojima T, Harita Y, Furuyama M, Sugawara N, Ishizuka K, Horita S, Kajiho Y, Miura K, Igarashi T, Hattori M, Kitanaka S: LMX1B mutation with residual transcriptional activity as a cause of isolated glomerulopathy. *Nephrol Dial Transplant* 2014; 29: 81-88.
 - 47) Gee HY, Ashraf S, Wan X, Vega-Warner V, Esteve-Rudd J, Lovric S, Fang H, Hurd TW, Sadowski CE, Allen SJ, Otto EA, Korkmaz E, Washburn J, Levy S, Williams DS, Bakkaloglu SA, Zolotnitskaya A, Ozaltin F, Zhou W, Hildebrandt F: Mutations in EMP2 cause childhood-onset nephrotic syndrome. *Am J Hum Genet* 2014; 94: 884-890.
 - 48) Gee HY, Zhang F, Ashraf S, Kohl S, Sadowski CE, Vega-Warner V, Zhou W, Lovric S, Fang H, Nettleton M, Zhu JY, Hoefele J, Weber LT, Podracka L, Boor A, Fehrenbach H, Innis JW, Washburn J, Levy S, Lifton RP, Otto EA, Han Z, Hildebrandt F: KANK deficiency leads to podocyte dysfunction and nephrotic syndrome. *J Clin Invest* 2015; 125: 2375-2384.
 - 49) Okamoto K, Tokunaga K, Doi K, Fujita T, Suzuki H, Katoh T, Watanabe T, Nishida N, Mabuchi A, Takahashi A, Kubo M, Maeda S, Nakamura Y, Noiri E: Common variation in GPC5 is associated with acquired nephrotic syndrome. *Nat Genet* 2011; 43: 459-463.
 - 50) Gbadegesin RA, Adeyemo A, Webb NJ, Greenbaum LA, Abeyagunawardena A, Thalgahagoda S, Kale A, Gipson D, Srivastava T, Lin JJ, Chand D, Hunley TE, Brophy PD, Bagga A, Sinha A, Rheault MN, Ghali J, Nicholls K, Abraham E, Janjua HS, Omoloja A, Barletta GM, Cai Y, Milford DD, O'Brien C, Awan A, Belostotsky V, Smoyer WE, Homstad A, Hall G, Wu G, Nagaraj S, Wigfall D, Foreman J, Winn MP; Mid-West Pediatric Nephrology Consortium: HLA-DQA1 and PLCG2 are candidate risk loci for childhood-onset steroid-sensitive nephrotic syndrome. *J Am Soc Nephrol* 2015; 26: 1701-1710.

Pathogenic mechanism of childhood idiopathic nephrotic syndrome

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Pathogenic mechanism of childhood nephrotic syndrome has been investigated for decades, and congenital nephrotic syndrome and early onset steroid resistant nephrotic syndrome has been found to mainly be attributed to podocyte dysfunction. Although many hypotheses regarding the pathogenesis of minimal change nephrotic syndrome (MCNS) including immunological abnormalities and podocyte dysfunction have been proved to be valid, the common definitive cause of MCNS has not been identified. Here, different hypotheses regarding the cause of MCNS are reviewed, and the difficulty in resolving the mechanism is discussed.

Key words: idiopathic nephrotic syndrome, minimal change nephrotic syndrome, podocyte

Biallelic Mutations in Nuclear Pore Complex Subunit *NUP107* Cause Early-Childhood-Onset Steroid-Resistant Nephrotic Syndrome

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The nuclear pore complex (NPC) is a huge protein complex embedded in the nuclear envelope. It has central functions in nucleocytoplasmic transport, nuclear framework, and gene regulation. Nucleoporin 107 kDa (*NUP107*) is a component of the NPC central scaffold and is an essential protein in all eukaryotic cells. Here, we report on biallelic *NUP107* mutations in nine affected individuals who are from five unrelated families and show early-onset steroid-resistant nephrotic syndrome (SRNS). These individuals have pathologically focal segmental glomerulosclerosis, a condition that leads to end-stage renal disease with high frequency. *NUP107* is ubiquitously expressed, including in glomerular podocytes. Three of four *NUP107* mutations detected in the affected individuals hamper *NUP107* binding to *NUP133* (nucleoporin 133 kDa) and *NUP107* incorporation into NPCs in vitro. Zebrafish with *nup107* knockdown generated by morpholino oligonucleotides displayed hypoplastic glomerulus structures and abnormal podocyte foot processes, thereby mimicking the pathological changes seen in the kidneys of the SRNS individuals with *NUP107* mutations. Considering the unique properties of the podocyte (highly differentiated foot-process architecture and slit membrane and the inability to regenerate), we propose a “podocyte-injury model” as the pathomechanism for SRNS due to biallelic *NUP107* mutations.

Introduction

Nephrotic syndrome (NS) is a renal disease caused by disruption of the glomerular filtration barrier, which results in massive proteinuria, hypoalbuminemia, and dyslipidemia. Idiopathic NS occurs in 16/100,000 children.¹ Most children with idiopathic NS respond well to steroids, but 10%–20% of affected children are categorized as having steroid-resistant NS (SRNS).^{2–6} SRNS is a clinically and genetically heterogeneous renal disorder that might have an immunological, structural, or functional etiology.^{2,5,7–9} Higher rates of genetic delineation are expected in early-onset SRNS.⁷ Clinical differences in SRNS have been suggested to depend on its age of onset.⁷ Current medical management and prognosis in NS are based largely on the histological diagnosis. Effective SRNS treatments are not well established, and renal transplantation is eventually required. Importantly, 63%–73% of those with childhood-onset SRNS show pathologically focal segmental glomeru-

losclerosis (FSGS), which carries a great risk of progression to end-stage renal disease (ESRD).^{1,6,8,10} To date, at least 27 genes are associated with SRNS, thereby expanding our knowledge of the pathomechanisms involved in SRNS and podocyte development and function.¹¹ Although SRNS is the leading cause of ESRD in children worldwide, approximately 70% of those with childhood-onset SRNS are genetically uncharacterized.^{7,11} We describe here an additional genetic cause of early-onset SRNS and propose its possible pathomechanism.

Material and Methods

Human Subjects

A total of 18 families (10 with affected siblings and 8 with a single affected individual) who lack any known genetic causes of SRNS (in 27 known genes) were recruited to this study. They presented with non-syndromic early-onset SRNS with onset ages between 1 and 11 years. The clinical aspects of 7 of the 18 families have

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<http://dx.doi.org/10.1016/j.ajhg.2015.08.013>. ©2015 by The American Society of Human Genetics. All rights reserved.



been described previously.¹² Affected individuals were resistant to standard steroid therapy but were partially responsive to immunosuppressive drugs. At least ten affected individuals in eight families underwent renal transplants and have had no recurrence of SRNS to date. All samples were collected after written informed consent was obtained. The study protocol was approved by the institutional review boards of Yokohama City University School of Medicine, Kansai Medical University, RIKEN, Tokyo Women's Hospital, and Kobe University.

DNA Extraction

Peripheral-blood leukocytes or saliva from affected individuals and their families was collected. Genomic DNA was extracted with a QIAamp DNA Blood Max Kit (QIAGEN) or Oragene DNA (DNA Genotek) according to the instructions of each manufacturer.

Whole-Exome Sequencing and Informatics Analyses

Whole-exome sequencing (WES) was performed on affected individuals (one individual from each family) and their parents when the samples were available, as reported previously.¹³ In brief, 3- μ g samples of genomic DNA were sheared with the Covaris S2 system (Covaris); genome partitioning was performed with SureSelect Human All Exon V5 (Agilent Technology) according to the manufacturer's instructions. Prepared samples were run on a HiSeq 2000 instrument (Illumina) with 101-bp paired-end reads and 7-bp index reads. The sequence reads were mapped to the human reference sequence (GRCh37) by Novoalign 3.00. Next, PCR duplication and variant calls were processed by Picard and the Genome Analysis Toolkit. Ten of the 18 families have multiple affected children, suggesting the autosomal-recessive model, in which homozygous or compound-heterozygous variants are focused in each affected individual. Genetic variants in exons and canonical splice sites (± 2 bp) with a minor allele frequency (MAF) of >0.005 in the NHLBI Exome Sequencing Project Exome Variant Server (EVS), Exome Aggregation Consortium (ExAC) Browser, Human Genetic Variation Database (HGVD, which is a public exome database for the Japanese population), or in-house Japanese exome data ($n = 575$) were removed from the candidates. Genes that harbor recessive variants detected commonly in two or more probands were selected. Candidate recessive variants were checked in each family by Sanger sequencing for confirmation that such variants co-segregated with the disease.

Haplotype Analysis

To determine the haplotype associated with c.2492A>C (p.Asp831Ala), which was found commonly in the five families, we amplified samples of genomic DNA or whole-genome-amplified DNA with 13 microsatellite markers (*D12S364*, *S12S310*, *D12S1617*, *D12S345*, *D12S85*, *D12S368*, *D12S83*, *D12S326*, *D12S351*, *D12S346*, *D12S78*, *D12S79*, and *D12S86*) from the ABI PRISM Linkage Mapping Set (Life Technologies). The PCR products were run on a 3500xl Genetic Analyzer (Life Technologies) and analyzed with GeneMapper 5 software (Life Technologies). Additionally, informative SNPs were chosen from the WES data for each affected individual and used thereafter for constructing haplotype blocks.

Expression of Human *NUP107*

NUP107 (nucleoporin 107 kDa; GenBank: NM_020401.2; MIM: 607617) expression in human embryos and adults was checked by a TaqMan Gene Expression Assay with two probe sets

(Hs00914854_g1 and Hs00220703_m1 from Life Technologies) internally standardized by beta actin (Life Technologies). cDNA from human fetal and adult tissues was purchased from Clontech. qPCR was performed by a Rotor-Gene Q instrument (QIAGEN), the data from which was analyzed by the $\Delta\Delta C_t$ method with Rotor-Gene 6000 Series software (QIAGEN). The experiments were done in duplicate. The expression level of each tissue represents the mean value of the duplicates.

Histopathology and Transmission Electron Microscopy on Samples from Individuals with Early-Onset SRNS

We stained 3- μ m-thick sections cut from paraffin-embedded biopsied kidney tissues with H&E, periodic acid-Schiff stain, and periodic acid methenamine silver stain according to standard methods. For transmission electron microscopy, 1-mm renal-biopsy specimen cubes were fixed in 2% phosphate-buffered glutaraldehyde (pH 7.3) at room temperature, dehydrated in an alcohol gradient, and embedded in Epon-Araldite resin. Sections of 1- μ m thickness were cut with an ultra-microtome (Ultracut UCT, Leica), stained with toluidine blue, and examined with a light microscope. Ultrathin sections (60–90 nm) stained by lead citrate were examined with a JEM1011 transmission electron microscope (JEOL). The TUNEL method was used to detect apoptotic cells on tissue sections with an in situ apoptosis detection kit (Takara) according to the manufacturer's instruction.

Immunofluorescence Microscopy

We deparaffinized and rehydrated 3- μ m-thick paraffin sections of a necropsy specimen and then autoclaved them in target retrieval solution (S1700, Dako) for 15 min at 105°C. The sections were subjected to immunofluorescence labeling with primary antibodies including rabbit anti-NUP107 mAb (1.5 μ g/ml, EPR12241, ab182559, Abcam), mouse anti-WT1 mAb (1:100, WT49, NCL-L-WT1-562, Leica), and mouse anti-Ezrin mAb (1:500, 3C12, E8897, lot 102K4824, Sigma-Aldrich). Normal rabbit and mouse immunoglobulins (IgGs) (sc-2027 [lot L1212] and sc-2025 [lot H1512], respectively, Santa Cruz) were used for negative controls. The CSAII kit (K1497, DAKO) was used for signal amplification of WT1, and other primary antibodies were visualized with Alexa555-conjugated anti-rabbit (1 μ g/ml) or Alexa647-conjugated anti-mouse IgG (2 μ g/ml) secondary antibodies (A21429 or A21236, respectively, Life Technologies), and then samples were mounted with ProLong Gold antifade reagent (P36930, Life Technologies). Single optical sections were acquired at 16-bit data depth with a confocal microscope system (AxioImager.Z1 microscope with LSM 700 laser scanner, Carl Zeiss) equipped with a C-Apochromat water immersion objective (40 \times , 1.2 numerical aperture [NA], Carl Zeiss); images were arranged with Photoshop CS5 (Adobe Systems).

Expression Vectors

Mammalian expression vectors were prepared with the Gateway system (Life Technologies). The *NUP107* open reading frame was amplified by PCR with human cDNA derived from a human lymphoblastoid cell line. The PCR product was introduced into the Gateway pDONR221 vector (Life Technologies), and its sequence was confirmed by Sanger sequencing. For mutagenesis, a QuickChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies) was used. After confirming appropriate mutagenesis, we performed LR recombination to create a mammalian expression

vector (pcDNA-DEST53, Life Technologies) to produce N-terminally GFP-fused NUP107 proteins. Among four *NUP107* mutations observed in this cohort, c.969+1G>A was mimicked by c.969_970insTAG, which created the nonsense codon just after the mutation (p.Asp324*). Whereas two truncating mutations (c.969+1G>A and c.1079_1083delAAGAG [p.Glu360Glyfs*6]) are thought unlikely to be present in vivo because of nonsense-mediated decay, these constructs were used as controls for the binding loss, given that C-terminally truncated proteins reportedly lose the NUP107-NUP133 interaction.¹⁴

Cell-free Protein Synthesis and In Vitro Pull-Down Assays

In vitro transcription and cell-free protein synthesis were performed as described previously.^{15,16} In vitro transcription templates for wild-type or mutant *NUP107* were amplified by split-primer PCR. For generation of transcription templates, the first PCR was performed with 50 ng/μl of each plasmid, 100 nM of the S1 common primer (5'-CCACCCACCACCACCAACAAAAAG CAGGCTATG-3'), and 100 nM of the vector-specific reverse primer (5'-ATCTTTTCTACGGGGTCTGA-3'). The second PCR was performed with the first PCR product as a template with 100 μM of the SPu primer (5'-GCGTAGCATTAGGTGACACT-3'), 100 μM of the vector-specific reverse primer (5'-ACGTTAAGGGATTTGGT CA-3'), and 1 μM of either the deSP6-E02-FLAG-tagged primer or the biotin-ligation site (bls) primer for the addition of the nucleotide sequences of the FLAG tag or the bls tag, respectively (FLAG tagged: 5'-GGTGACACTATAGAATCACCTATCTCTACACAAA ACATTTCCTACATACAACCTTCACTTCCTATTATGGACTACAA GGATGACGATGACAAGCTCCACCCACCACCACCAATG-3'; bls tagged: 5'-GGTGACACTATAGAATCACCTATCTCTACACAAA ACATTTCCTACATACAACCTTCACTTCCTATTATGGGCTGACGACATCTTCGAGGCCAGAAAGATCGAGTGGCACGAACTCC ACCCACCACCACCAATG-3').

An ENDEXT Wheat Germ Expression Kit (CellFree Sciences) was used for cell-free protein synthesis according to the manufacturer's instructions for the bilayer translation method. Biotinylated proteins were produced as described previously.¹⁷

Biotinylated wild-type or altered *NUP107* was mixed with FLAG-NUP133 (nucleoporin 133 kDa; GenBank: NM_018230.2; MIM: 607613) in lysis buffer containing 25 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 2% Triton X-100, 1 mM DTT, and 10 mg/ml BSA. After incubation for 1 hr at 26°C, streptavidin MagneSphere beads (Promega) were added, and the mixture was incubated for 30 min at room temperature. After three washes with lysis buffer, bound proteins were eluted from the beads with 20 μl of 2× SDS sample buffer. Bound proteins were separated by SDS-PAGE followed by immunoblotting with an anti-FLAG antibody (Sigma-Aldrich) or a Streptavidin-HRP conjugate (GE Healthcare). Proteins on the blot were detected with Immobilon Western Chemiluminescent HRP Substrate (Millipore) and FluorChem FC2 (Alpha Innotech) in accordance with the protocol from each manufacturer.

Immunoprecipitation

The cell lysate used for immunoprecipitation was prepared according to a method reported previously^{18,19} with a slight modification. In brief, HeLa cells were transfected with the wild-type or altered N-terminally GFP-fused *NUP107* construct by Viafect (Promega) according to the manufacturer's instructions. The cells were lysed with lysis buffer containing 10 mM Tris-HCl (pH 7.4),

400 mM NaCl, 1% Triton X-100, 2 mM EDTA, 1 mM DTT supplemented with complete proteinase inhibitor cocktail (Roche Diagnostics GmbH), and PhosSTOP (Roche Diagnostics); sonicated; and then incubated for 30 min at 4°C. For debris removal, the crude lysate was centrifuged at 20,630 × g for 20 min at 4°C. After collection, the supernatant was diluted 3.75× in dilution buffer (10 mM Tris-HCl [pH 7.4], 2 mM EDTA, 1 mM DTT, complete proteinase inhibitor cocktail, and PhosSTOP). For immunoprecipitation of the GFP-fused *NUP107*, mouse anti-GFP antibody (11-814-460-001, Roche Diagnostics) and Protein G Sepharose beads (17-0618-01, GE Healthcare) were added. After incubation for 2 hr at 4°C, the beads were washed with wash buffer (lysis buffer diluted 3.75× in dilution buffer). After the protein-bound beads were boiled, they were run on an SDS-PAGE gel and transferred to a polyvinylidene fluoride membrane (Millipore). Membranes prepared in this manner were incubated in 0.2% Casein in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) for blocking. The membrane was probed with rabbit anti-GFP primary antibody (598, MBL) diluted at 1:1,000 and mouse anti-NUP133 (M00055746-M01, Abnova) diluted at 1:500 followed by secondary antibodies HRP-rabbit anti-rat IgG (A5795, Sigma-Aldrich) and HRP-goat anti-mouse IgG (170-6516, Bio-Rad) both diluted at 1:3,000 with 0.2% Casein in TBS-T. For obtaining protein signals, Immobilon Western Chemiluminescent HRP Substrate (Millipore) was used as a chemiluminescence substrate.

Subcellular Localization of NUP107

HeLa cells cultured in DMEM (Life Technologies) containing 10% fetal bovine serum (Sigma-Aldrich) at 37°C in an atmosphere of 5% CO₂ on poly-L-lysine-coated coverslips (Wako) were transfected with the wild-type or altered N-terminally GFP-fused *NUP107* vector with the use of Viafect (Promega). After incubation for 48 hr, the cells were washed with pre-warmed PBS at 37°C and then fixed with pre-warmed 2% paraformaldehyde (Wako) in PBS at 37°C for 10 min. The cells were treated with 0.5% Triton X-100 in PBS for 2.5 min and then incubated with 5% normal goat serum (NGS, Merck Millipore) in PBS for 1 hr. After blocking, the cells were reacted with the primary antibody (MAB414 [mouse anti-nuclear pore complex (NPC) proteins], MMS-120P, Covance) diluted at 1:3,000 in 1% NGS in PBS for 2 hr, washed with PBS, and then reacted with the secondary antibody (Alexa Fluor 594 goat anti-mouse IgG, A11032, Life Technologies) in 1% NGS in PBS for 2 hr. After staining, the cells were mounted in paraphenylenediamine solution (80% glycerol in PBS and 1 mg/ml paraphenylenediamine, 11873580001, Roche Diagnostics). Images were captured with a DeltaVision microscope (Applied Precision) equipped with a Plan Apo objective lens (100×, 1.35 NA, Olympus) and a Cool Snap HQ2 CCD camera (Photometrics).

Zebrafish Knockdown by Microinjection of Morpholino Oligonucleotides

The antisense morpholino oligonucleotides (MOs) for *nup107* translation blocking (TB) (5'-AAGTCTGACTCCATTCATATT GTC-3')²⁰ and for *nup107* splice blocking (SB) (5'-ATACATTAA AGTCCACCTCTCTGAC-3') and a standard MO control (5'-CCT CTTACCTCAGTTACAATTATA-3') obtained from Gene Tools were injected into 1- to 2-cell-stage embryos, each at a final concentration of 0.25 mM. The experiment was authorized by the Institutional Committee for Fish Experiments at the National Research Institute of Fisheries Science.