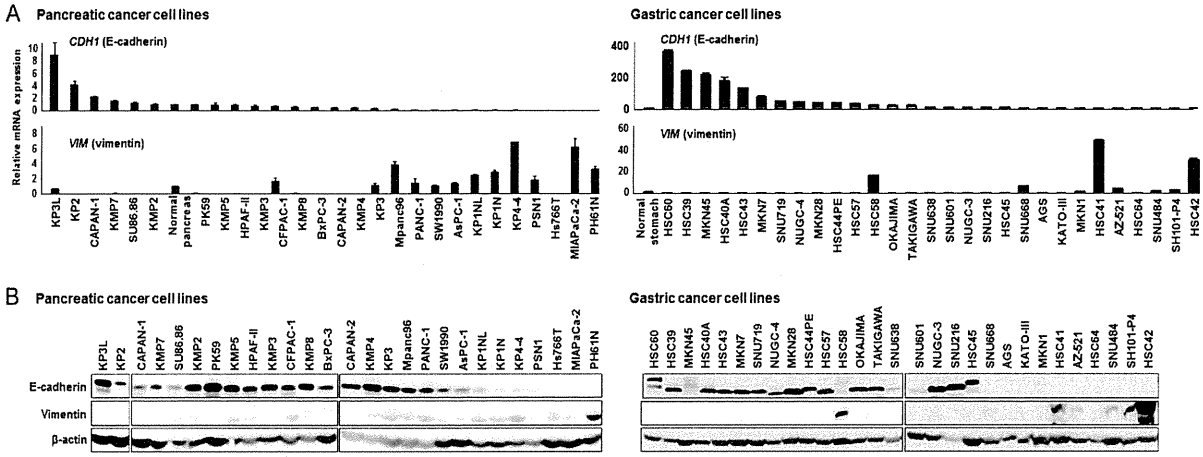

図1. 膵がん細胞株27株 (左)、胃がん細胞株27株 (右)のE-カドヘリン、ビメンチン発現量　A. *mRNA*の発現量　B. タンパク質の発現量

膵がん細胞株 27 株、胃がん細胞株 29 株を用いて、E-カドヘリン、ビメンチンの発現を定量 RT-PCR およびウエスタンブロッティング法により検討した。多くの細胞株は、既報にあるような E-カドヘリンとビメンチンの発現が逆相関していた (図 1)。

この解析において、E-カドヘリン、ビメンチンの両方を発現している細胞株は、EMT 可塑性を有する可能性があると仮説を立て、EMT 可視化システムに用いる細胞株として選出した (膵がん: Panc1, KP1N、胃がん: MKN)。Panc1 は、既に EMT 可塑性を有する細胞として知られており、EMT 研究の代表的なモデル細胞である。選出した細胞株の EMT 可塑性の評価は、*miR-200* ファミリーの導入および TGF-β を処理によって行った。その結果、選出したすべての細胞株が、EMT 可塑性を有することを確認した (図 2)。

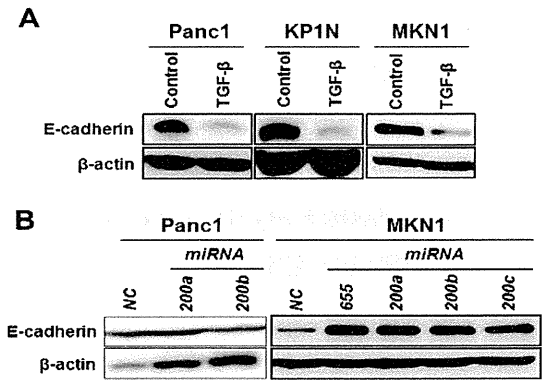

図2. EMT可塑性評価　A. TGF-β添加の際のEMT誘導評価　B. EMT抑制性*miRNA*導入時のMET誘導評価

EMT 可視化システムの有用性の検討

EMT 可視化システムは、*CDH1* および *VIM* のプロモーター活性を指標としているため、我々は *CDH1*、*VIM* のプロモーター領域を挿入したレポーターコンストラクトを作製した (PEcadZsG, PVimRFP)。Panc1, KP1N には PEcadZsG を、MKN1 には PVimRFP を導入し、安定発現株を樹立した。

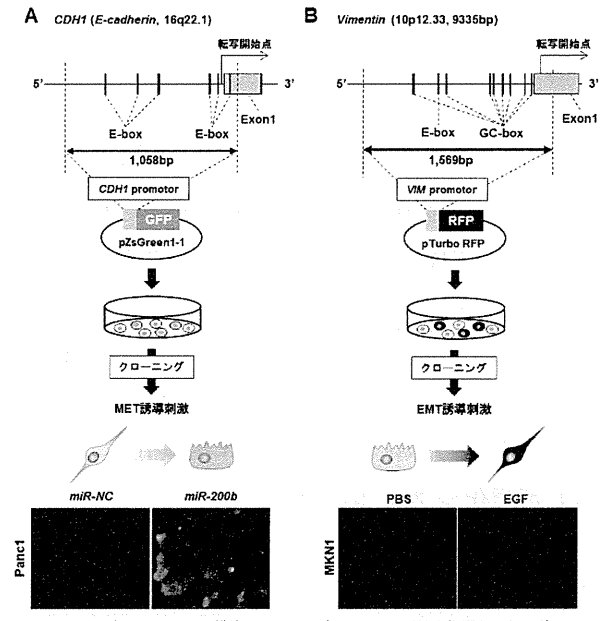

図3. EMT可視化システムの構築　A. *CDH1*プロモーター活性を指標としたレポーターコンストラクトの作製とGFP応答性の確認 (Panc1)　B. *VIM*プロモーター活性を指標としたレポーターコンストラクトの作製とRFP応答性の確認 (MKN1)

PEcdZsG を導入した Panc1, KP1N (pEcadZsG -Panc1, -KP1N)では、*miR-200b* の過剰発現により、コントロールに比して GFP 蛍光タンパク質の発現上昇を認め、それに伴うように *CDH1* の転写レベルも増加していた(図 3)。また同様に PVimRFP

を導入した MKN1 (PVimRFP-MKN1)では、EGF 処理により、コントロールに比して RFP 蛍光タンパク質の上昇を認め、同時に *VIM* の転写レベルも増加していた。上記結果より、本システムが正常に機能していることが明らかとなった。

miRNA ライブラリーを用いた機能的スクリーニング

まず、我々は上記システムを用いた機能的スクリーニングに当たり、EMT 研究において最も使用されている細胞株である Panc1 (PEcadZsG-Panc1)を優先的に解析した。機能的スクリーニングには、470 種類の *miRNA* を搭載した Pre-miR™ *miRNA* Precursor Library-Human V3 を用いて、新規 EMT 抑制性 *miRNA* の探索を行った。ライブラリー導入後 96 時間の時点で、それぞれの細胞が発現する GFP の量を吸光度計により定量化した後、細胞増殖能試験を行い、細胞増殖による補正を行った(**図 4**)。その結果、*miR-200* ファミリーを含む複数の *miRNA* が EMT 抑制性候補 *miRNA* として抽出された。その中でも特に E-カドヘリンの発現を誘導した *miR-655* の機能解析を進めた。

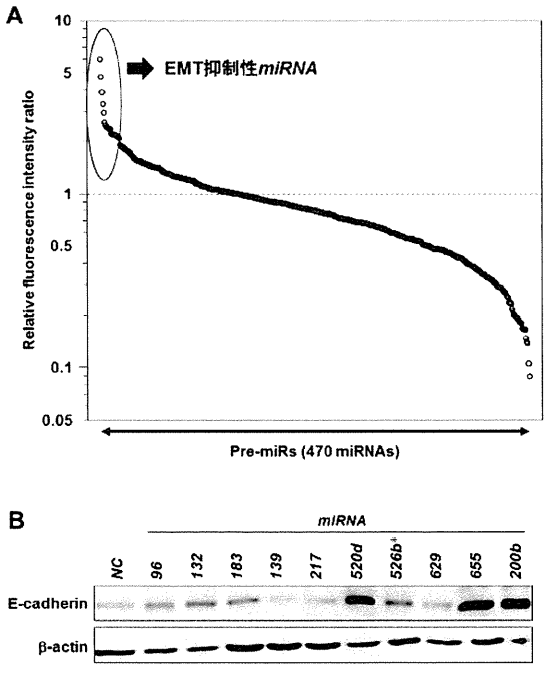

図4. Pre-miRTM miRNA Precursor Library-Human V3を用いた機能的スクリーニング　A. 470種類の*miRNA*導入時の蛍光値グラフ　B. EMT抑制性候補*miRNA*導入によるE-カドヘリン発現変化

miR-655 は、*ZEB1, TGFBR2* を標的にすることにより MET を誘導する

我々は、*miR-655* がどのように MET を誘導するのかを探索するため、*miRNA* 標的探索公共データベースである TargetScan を用いて *miR-655* が直接制御する遺伝子の抽出を行った。抽出した遺伝子の中には、EMT に重要な TGF-β 経路に属する *ZEB1* および *TGFBR2* が含まれていたので、ルシフェラーゼレポーターアッセイを用いて、*miR-655* が上記遺伝子の 3'UTR に結合するか検討した。その結果、*miR-655* は、直接的に *ZEB1* および *TGFBR2* の発現抑制を行うことにより、E-カドヘリンの発現が上昇し、MET を誘導することが明らかとなった (**図 5**)。

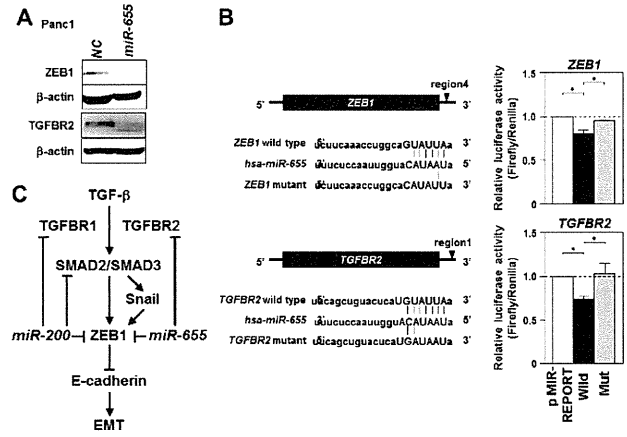

図5. *miR-655*は、*ZEB1,TGFBR2*に直接結合し発現制御を行う。　A. *miR-655*導入時の*ZEB1, TGFBR2*の発現量　B. ルシフェラーゼレポーターアッセイを用いた結合実験　C. *miR-655*を含めたEMT遺伝子ネットワークの概念図

miR-655 の発現は、OSCC および ESCC 臨床検体において発現低下しており、ESCC の予後予測因子としての可能性を持つ

次に、ヒトがん臨床検体を用いて *miR-655* の発現と臨床病理学的因子との解析を行った。対象としたのは、OSCC (23 症例)と ESCC (29 症例)臨床検体であり、非がん部とがん部での *miR-655* の発現を検討した。その結果、OSCC では 60.9% (14/23)、ESCC では 44.8% (13/29)の頻度でがん部における *miR-655* の顕著な発現低下を認めた。さらに、ESCC では *miR-655* 低発現群は、高発現群に比して予後不良であることが明らかとなり、*miR-655* が予後予測因子となる可能性が示唆された(**図 6**)。

関連 *miRNA* が同定されることにより、浸潤・転移を標的とした核酸医薬の開発ならびに *miRNA* デリバリー技術の確立によるその臨床応用の可能

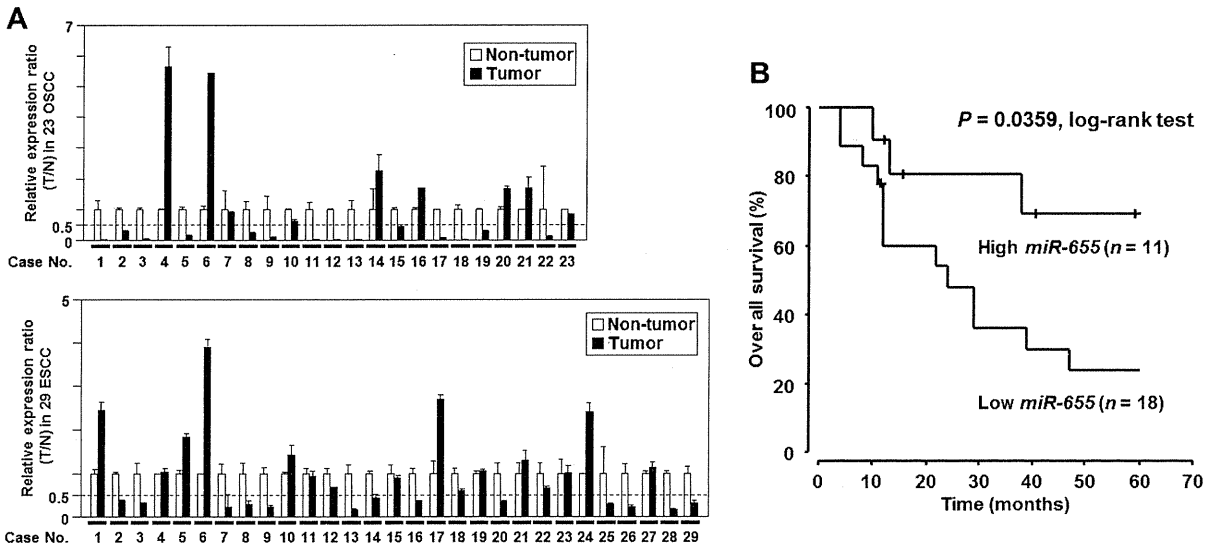

図6. *miR-655*の発現は、ESCCの予後因子となりえる。 A. OSCC, ESCCにおける*miR-655*の発現量 B. ESCCにおける*miR-655*発現ステータスで分類した予後曲線

D. 考察

EMT は、浸潤・転移を促進するがん悪性化の原因のひとつであるが、その動的な性質から解析が困難であり、経時的に観察可能なスクリーニング系は限られていた。本研究では、EMT 関連遺伝子のプロモーター活性依存的に発現する蛍光タンパク質を用いて EMT の動的変化を可視化するシステムを構築したことにより、網羅的なスクリーニングから新規 EMT 関連遺伝子の同定を可能にした。今回の結果では、Panc1 を用いた結果を示したが、KP1N および MKN1 においても機能的スクリーニングを施行している。*VIM* のプロモーター活性を指標とした MKN1 では、既に 328 種類の *miRNA* を搭載した Pre-miR™ *miRNA* Precursor Library-Human V2 を用いて、EMT 促進性 *miRNA* の候補の絞込みが終了しており、複数の候補 *miRNA* を対象に機能解析へと進んでいる。この候補 *miRNA* の中の一つは、Wnt 経路の制御因子として重要な遺伝子を抑制することにより、βカテニンの核内移行を促進し、EMT を誘導させることが明らかとなってきている。このように新規 EMT 性にも期待できると考える。

本システムは、がん種を問わず EMT 可塑性を有する細胞であれば応用可能であり、また、*miRNA* ライブラリーだけではなく各種ライブラリー(*shRNA*, *cDNA*, 化合物)を用いた解析にも有用であると考えられる。特に、本システムは細胞ベースのアッセイ系であるため、化合物ライブラリーを用いることにより、がんでは適応外の既存承認薬の再配置 (ドラッグリポジショニング)を行うことが可能であり、薬の開発にかかる資金・時間を大幅に削減でき、かつ有用な分子を見出せる可能性を秘めている。

E. 結論

今回、我々は膵がん、胃がんを対象に EMT 可視化システムの構築を実現させ、機能的スクリーニングを施行することにより、新規 EMT 抑制性 *miR-655* を同定した。本システムは、各種ライブラリーと組み合わせることにより、EMT を基軸とした浸潤・転移に関わる遺伝子ネットワークを描出することが可能な画期的なものであり、浸潤・転移を標的とした新規治療法確立の足がかりに

なると期待される。

以上の研究は、代表者と村松智輝、谷中淑光により実施された。

F．健康危険情報

なし

G．研究発表

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H．知的財産権の出願・登録状況
（予定を含む。）

1. 特許取得

1. 発明の名称 ： 卵巣癌の検出方法及び抑制方法
分割出願番号 ： 特願 2012-209426
（出願日：2012/09/24）
基礎出願番号 ： 特願 2007-143111
（出願日：2007/05/30）
登録番号：特許第 5645089 号
（登録日：2014/11/14）
発明者 ： 稲澤譲治、井本逸勢、菊池良子
出願人 ： 富士フイルム株式会社、国立大学法人東京医科歯科大学

2. 発明の名称： 核酸マイクロアレイのデータ補正方法
出願番号：200980128809.X
（出願日： 2009/05/27）
公開番号：CN102105597
（公開日： 2011/06/22）
登録番号：200980128809.X
（登録日： 2014/07/02）
発明者： 氏原大、金原秀行、稲澤譲治、井本逸勢
出願人： 富士フイルム株式会社、国立大学法人東京医科歯科大学

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出願番号：特願 2010-041825
（出願日：2010/02/26）
公開番号：特開 2011-177034
（公開日： 2011/09/15）
登録番号：特許第 5557139 号
（登録日：2014/06/13）
発明者： 稲澤譲治、井本逸勢、春木茂男
出願人： 富士フイルム株式会社、国立大学法人東京医科歯科大学

2. 実用新案登録
なし

3.その他
なし

Ⅲ.　学会等発表実績

学会等発表実績

委託業務題目「スキルスがんにおける癌幹細胞　悪性形質獲得機構に関する研究」
機関名　国立大学法人東京医科歯科大学　大学院医歯学総合研究科　消化管先端治療学

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The acquisition of cancer stemness by Atoh1 in colitis associated cancer(ポスター)	Tsuchiya K,Fukushima K, Watanabe M	Barcelona,Spain(10th Congress of ECCO - Inflammatory Bowel Diseases 2015)	2015 年 2 月 20 日	国外
Long-term stimulation with cytokines acquires irreversible accumulation of NF-_B signaling in colonic epithelial cells.(ポスター)	Hibiya S, Tsuchiya K, Fukushima K, Hayashi R, Horita N, Oshima S, Okamoto R, Nakamura T, Watanabe M	Barcelona,Spain(10th Congress of ECCO - Inflammatory Bowel Diseases 2015)	2015 年 2 月 19 日 -21 日	国外
Human alpha-defensin 6 regulated by both atoh1 and beta-catenin might be the pathogeneses of crohn's disease.(ポスター)	Hayashi R, Tsuchiya K, Hibiya S, Fukushima K, Horita N, Okada E, Araki A, Ohtsuka K, Watanabe M	Vienna, Austria(UEGW2014)	2014 年 10 月 22 日	国外
Notch Signaling and TNF-alpha synergistically promotes intracellular protein accumulation of olfm4 in the inflamed mucosa of ulcerative colitis.(ポスター)	Ito G, Okamoto R, Shimizu H, Fujii S, Nakata T, Suzuki K, Tsuchiya K, Nakamura T, Watanabe M	Vienna, Austria(UEGW2014)	2014 年 10 月 21 日	国外
Long-term stimulation with cytokines leads to irreversible NF-kB signaling activation in colonic epithelial cells.(口頭)	Hibiya S, Tsuchiya K, Watanabe M	Yokohama(The 73rd Annual Meeting of the Japanese Cancer Association)	2014 年 9 月 25 日	国内
Innate immune spiral of intestinal epithelial cells by the longterm inflammation.(口頭)	Tsuchiya K , Hibiya S, Watanabe M	Yokohama(The 73rd Annual Meeting of the Japanese Cancer Association)	2014 年 9 月 25 日	国内
Establishment of the gene transduction into the primary intestinal organoid identified the subpopulation of the stem cells in a crypt.(口頭)	Horita N, Tsuchiya K, Hayashi R, Fukushima K, Hibiya S, Fukuda M, Kano Y, Mizutani T, Nemoto Y, Yui S, Okamoto R, Nakamura T, Watanabe M	Fukuoka(12th Stem Cell Research Symposium)	2014 年 5 月 31 日	国内
臨床検体を用いた小腸病態解析と再生医療への展開(口頭)	土屋　輝一郎	東京(第１２回線維化病態研究会)	2015 年 2 月 9 日	国内
Continuous stimulation with cytokines acquires irreversible accumulation of NF-_B signaling in primary colonic epithelial cells.(ポスター)	日比谷 秀爾、土屋 輝一郎、福島 啓太、林 亮平、堀田 伸勝、加納 嘉人、大島 茂、岡本 隆一、中村 哲也、渡辺 守	東京(がん研究分野の特性等を踏まえた支援活動 公開シンポジウム)	2015 年 1 月 27-28 日	国内
腸管上皮初代培養細胞を用いた体外病態モデルの構築 (口頭)	土屋輝一郎、堀田伸勝、福島啓太、林　亮平、日比谷秀爾、水谷知裕、大島　茂、永石宇司、岡本隆一、中村哲也、大塚和朗、渡辺　守	東京(厚生労働科学研究費委託費難治性疾患等実用化研究事業 「独自の体外病態モデルによる難治性炎症性腸疾患の革新的治療薬開発に関する研究」)	2015 年 1 月 23 日	国内
クローン病病態解明を主眼としたα-defensin 発現制御機構解析(口頭)	林　亮平、土屋輝一郎、渡辺守	東京（開催場所：ホテル東京ガーデンパレス）(第 52 回 小腸研究会)	2014 年 11 月 15 日	国内
炎症性発癌大腸癌における Atoh1 蛋白発現と癌幹細胞形質獲得機構(口頭)	福島啓太、土屋輝一郎、渡辺守	神戸(第 22 回日本消化器関連学会週間)	2014 年 10 月 23 日	国内

発表した成果 (発表題目、口頭・ポスター発表の別)	発表者氏名	発表した場所 (学会等名)	発表した時期	国内・外の別
出血傾向のある放射線性腸炎に対するアルゴンプラズマ凝固法の検討(口頭)	松沢　優、竹中　健人、福田　将義、和田　祥城、岡田　英里子、土屋　輝一郎、荒木　昭博、大塚　和朗、渡辺　守	東京(帝京大学霞が関キャンパス（永田町）)(日本大腸検査学会総会)	2014年9月6日	国内
腸管上皮初代培養細胞を用いた体外病態モデルの構築 (口頭)	土屋輝一郎、堀田伸勝、福島啓太、林　亮平、日比谷秀爾、水谷知裕、大島　茂、永石宇司、岡本隆一、中村哲也、大塚和朗、渡辺　守	東京(厚生労働科学研究費委託費難治性疾患等実用化研究事業「独自の体外病態モデルによる難治性炎症性腸疾患の革新的治療薬開発に関する研究」)	2014年7月25日	国内
小腸生検検体を用いたクローン病病態解析(口頭)	林　亮平、土屋輝一郎、渡辺　守	東京(第100回日本消化器病学会総会)	2014年4月26日	国内
NSAIDs・抗血小板薬による小腸粘膜障害の病理学的検討(口頭)	岡田英理子、土屋輝一郎、渡辺守	東京(第100回日本消化器病学会総会)	2014年4月26日	国内
Exploring cancer-related miRNAs by function-based screening.(口頭)	稲澤譲治	ソウル（韓国）(international conference on the 19th Annual Meeting of Korean Society of cancer Prevention.)	2014年12月12日	国外
Exploring Tumor Suppressor microRNAs Silenced by Tumor-specific DNA Hyper-Methylation in Cancer.(口頭)	稲澤譲治	マニラ（フィリピン）(2014 17th　Annual Conference A-IMBN .st.Luke’s Medical Center-Global City Taguig)	2014年12月1日	国外
機能的スクリーニングによるがん抑制性マイクロRNAの探索と診断、治療への応用(口頭)	稲澤譲治	一橋講堂　学術総合センター（東京）(がん研究分野の特性等を踏まえた支援活動　公開シンポジウム)	2015年1月28日	国内
東京医科歯科大学疾患バイオリソースセンターの構築とバイオバンク事業(口頭)	稲澤譲治	札幌医科大学臨床第一講義室（北海道）(遺伝子解析研究審査委員会 研修会)	2014年12月19日	国内
進展するがんゲノミクス・エピゲノミクス研究・がん関連マイクロRNAの探索と診断・治療への応用(口頭)	稲澤譲治	タワーホール船堀（東京）(日本人類遺伝学会第59回大会シンポジウム)	2014年11月22日	国内
がん抑制性RNAの探索と同定. 第73回日本癌学会学術総会(口頭)	稲澤譲治	パシフィコ横浜(神奈川)（第73回日本癌学会学術総会)	2014年9月27日	国内
高転移腫瘍における統合的解析から同定された新規治療標的経路ハイプシンカスケードの解析(口頭)	村松智輝、小崎健一、井元清哉、山口類、津田均、森下真紀、河野辰幸、宮野悟、稲澤譲治	パシフィコ横浜(神奈川)（第74回日本癌学会学術総会)	2014年9月27日	国内
新規発癌機構であるchromothripsis生成機序の解明(口頭)	森下真紀、村松智輝、林深、平井百樹、数藤由美子、森山啓司、稲澤譲治	パシフィコ横浜(神奈川)（第75回日本癌学会学術総会)	2014年9月27日	国内
食道癌におけるLAPTM5の不活性化. 第73回日本癌学会学術総会(口頭)	ヌイラン・ミッシェル、井上純、河野辰幸、稲澤譲治	パシフィコ横浜(神奈川)（第76回日本癌学会学術総会)	2014年9月26日	国内
細胞ベースのリポーターアッセイを用いた胃癌でvimentinを発現上昇させるmiRNAの探索(ポスター)	谷中淑光、小崎健一、稲澤譲治	パシフィコ横浜(神奈川)（第77回日本癌学会学術総会)	2014年9月26日	国内
マイクロRNAを基盤としたNRF2活性化癌に対する診断・治療法の確立(口頭)	井上純、山本信祐、藤原直人、河野辰幸、小村健、小崎健一、稲澤譲治	パシフィコ横浜(神奈川)（第78回日本癌学会学術総会)	2014年9月25日	国内
p62/SQSTM1タンパク質の高発現は子宮体癌の悪性形質および不良な予後と相関する(口頭)	岩舘怜子、井上純、津田均、平沢晃、青木大輔、稲澤譲治	パシフィコ横浜(神奈川)（第78回日本癌学会学術総会)	2014年9月25日	国内
新規癌抑制性microRNAとしてのMir-634の同定(ポスター)	藤原直人、井上純、河野辰幸、小崎健一、稲澤譲治	パシフィコ横浜(神奈川)（第78回日本癌学会学術総会)	2014年9月25日	国内
東京医科歯科大学疾患バイオリソースセンターの構築とバイオバンク事業(口頭)	稲澤譲治	大手門学院大阪城スクエア（大阪）(大阪がん患者データベース研究会 第28回研究集会)	2014年9月13日	国内

発表した成果 (発表題目、口頭・ポスター発表の別)	発表者氏名	発表した場所 (学会等名)	発表した時期	国内・ 外の別
The potential of miR-634-mediated modulation for cancer therapy(口頭)	藤原直人、井上純、河野辰幸、小崎健一、稲澤譲治	蓼科グランドホテル滝の湯（長野(平成 26 年度文部科学省科学研究費補助金・新学術領域研究「がん研究分野の特性等を踏まえた支援活動」. がん若手研究者ワークショップ))	2014 年 9 月 5 日	国内
最新のがん研究の動向(口頭)	稲澤譲治	仙台市情報産業プラザ（宮城）(第 18 回日本がん分子標的治療学会学術集会.市民公開講座)	2014 年 6 月 28 日	国内
マイクロ RNA を基盤とした NRF2 活性化癌に対する診断・治療法の確立(ポスター)	井上純、稲澤譲治	仙台市情報産業プラザ（宮城）(第 18 回日本がん分子標的治療学会学術集会)	2014 年 6 月 26 日	国内
東京医科歯科大学におけるバイオバンク事業と今後の展望.(口頭)	稲澤譲治	岡山大学 Junko Fukutake Hall（岡山）(岡山大学大学院特別講演)	2014 年 6 月 17 日	国内
んと遺伝疾患のゲノム解析：最近の話題(口頭)	稲澤譲治	京都ブライトンホテル（京都）(京都小児科医会 専攻医・研修医合同講演会)	2014 年 4 月 19 日	国内

2．学会誌・雑誌等における論文掲載

掲載した論文（発表題目）	発表者氏名	発表した場所 (学会誌・雑誌等名)	発表した時期	国内・ 外の別
Associations of HLA class I alleles in Japanese patients with Crohn's disease.16(1):54-56	Oryoji D, Hisamatsu T, Tsuchiya K, Umeno J, Ueda S, Yamamoto K, Matsumoto T, Watanabe M, Hibi T, Sasazuki T	Genes Immun.	2015 年・1 月	国外
RIPK3 regulates p62-LC3 complex formation via the caspase-8-dependent cleavage of p62.456(1):298-304	Matsuzawa Y, Oshima S, Nibe Y, Kobayashi M, Maeyashiki C, Nemoto Y, Nagaishi T, Okamoto R, Tsuchiya K, Nakamura T, Watanabe M	Biochem Biophys Res Commun.	2015 年・1 月	国外
Fluorescent labelling of intestinal epithelial cells reveals independent long-lived intestinal stem cells in a crypt.454:493-499	Horita N, Tsuchiya K, Hayashi R, Fukushima K, Hibiya S, Fukuda M, Kano Y, Mizutani T, Nemoto Y, Yui S, Okamoto R, Nakamura T, Watanabe M	Biochem Biophys Res Commun.	2014 年・10 月	国外
Distinct expression patterns of Notch ligands, Dll1 and Dll4, in normal and inflamed mice intestine.2:e370; DOI 10.7717/peerj.370.	Shimizu H, Okamoto R, Ito G, Fujii S, Nakata T, Suzuki K, Murano T, Mizutani T,Tsuchiya K, Nakamura T, Hozumi K, Watanabe M	PeerJ	2014 年・5 月	国外
High expression of SQSTM1/p62 protein is associated with poor prognosis in epithelial ovarian cancer.46(2):295-301	Iwadate R, Inoue J, Tsuda H, Takano M, Furuya K, Hirasawa A, Aoki D, Inazawa J	Acta Histoche Cytochem	2015 年・2 月	国外
Overexpression of SMYD2 contributes to malignantoutcome in gastric cancer,112(2):357-64	Komatsu S, Ichikawa D, Hirajima S, Nagata H, Nishimura Y, Kawaguchi T, Miyamae M, Okajima W, Ohashi T, Konishi H, Shiozaki A, Fujiwara H, Okamoto K, Tsuda H, Imoto I, Inazawa J, Otsuji E	Br J Cancer.	2015 年・1 月	国外

Ⅳ.　研究成果の刊行物・別刷

Genes and Immunity (2014), 1–3

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ORIGINAL ARTICLE

Associations of *HLA* class I alleles in Japanese patients with Crohn's disease

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Previous studies have suggested that the human leukocyte antigen (HLA) is involved in the etiology of Crohn's disease (CD); however, few reports are available on the association between HLA class I antigens and CD in Japan. In this study, we performed association analysis of HLA class I antigens in CD using 208 Japanese patients and 384 healthy controls. We identified novel positive associations between CD and *HLA-A*02:01* (odds ratio (OR) = 1.64, $P = 0.016$) and *HLA-A*02:07* (OR = 2.31, $P = 0.0067$) and confirmed previously reported positive associations between CD and *HLA-Cw*14:02* (OR = 2.18, $P = 0.0021$) and *HLA-B*51:01* (OR = 1.70, $P = 0.033$). We also identified novel negative associations between CD and *HLA-A*24:02* (OR = 0.60, $P = 0.0047$) and *HLA-B*07:02* (OR = 0.38, $P = 0.0041$). Although the associations were not significant after full Bonferroni correction, we suggested that HLA class I genes have dual functions, susceptibility and resistance in controlling the development of CD.

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INTRODUCTION

Crohn's disease (CD) and ulcerative colitis, two main subtypes of inflammatory bowel disease, are chronic and relapsing inflammatory disorders of the gastrointestinal tract caused by an aberrant response of the intestinal immune system to commensal bacteria.[1,2] CD affects all regions of the gastrointestinal tract, most commonly the ileum and colon, whereas the inflammation in ulcerative colitis is confined to the colon. The pathological changes in CD are typically discontinuous and often transmural. By contrast, those in ulcerative colitis are continuous and confined to the mucosa and submucosa.[2] Although the prevalence of CD is lower (23.6/100 000 persons) in Japan compared with European populations, it has been increasing continuously over the past several decades in Japan and other Asian countries.[3]

Although their exact etiology remains unclear, genetic factors appear to contribute to the susceptibility to these diseases. The genetic component seems stronger in CD than in ulcerative colitis.[4] A statistically significant association of human leukocyte antigen (HLA) with CD has been widely reported, especially for the *HLA* class II antigens;[5–9] reports of this association include a meta-analysis by Stokkers *et al.*[10] However, the data from these analyses should be interpreted with caution because they include data from both serological and molecular typing of *HLA*, as well as studies on different ethnic groups. There have been a few reports on the association of *HLA* class I alleles with CD in the Japanese population; however, there have been no reports specifically on the association of *HLA-A* alleles with CD in Japan. To elucidate the role of *HLA* class I antigens in the etiology of CD, we investigated the genetic association between the *HLA-A*, *-C* and *-B* alleles and CD in this study.

RESULTS

Association of HLA class I antigens with CD

We analyzed 10 *HLA-A*, 12 *HLA-C* and 18 *HLA-B* alleles with frequencies >1.0%. Compared with the controls, the antigen frequencies of *HLA-A*02:01* (odds ratio (OR) = 1.64, $P = 0.016$), *HLA-A*02:07* (OR = 2.31, $P = 0.0067$), *HLA-Cw*14:02* (OR = 2.18, $P = 0.0021$), *HLA-B*46:01* (OR = 1.88, $P = 0.018$) and *HLA-B*51:01* (OR = 1.70, $P = 0.033$) were increased in patients with CD, whereas the antigen frequencies of *HLA-A*24:02* (OR = 0.60, $P = 0.0047$) and *HLA-B*07:02* (OR = 0.38, $P = 0.0041$) were decreased (Table 1). Thus, we identified three novel susceptibility alleles for CD, *HLA-A*02:01*, *HLA-A*02:07* and *HLA-B*46:01*, and we confirmed two previously reported susceptibility alleles for CD, *HLA-Cw*14:02* and *HLA-B*51:01*.[11] Furthermore, we identified two novel protective alleles for CD, *HLA-A*24:02* and *HLA-B*07:02*. However, it should be noted that these associations were not statistically significant after Bonferroni correction. Therefore, the associations newly found in our study need to be replicated using another sample set in future study.

Among the three novel susceptibility alleles, linkage disequilibrium (LD) is known to occur between *HLA-A*02:07* and *HLA-B*46:01* in the Japanese population. Conditional analyses showed that *HLA-A*02:07* affected the strength of the association between *HLA-B*46:01* and CD and vice versa (Table 2). This observation, along with the fact that the association for *HLA-A*02:07* was more significant than the association for *HLA-B*46:01* in the non-conditional analysis (Table 1), suggests that *HLA-A*02:07* is the primary susceptibility allele for CD.

The novel protective alleles, *HLA-A*24:02* and *HLA-B*07:02*, are also known to be in LD in the Japanese population. However, we observed that the associations between these alleles and CD were still significant even when conditioned on each other (Table 2).

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This observation suggests that *HLA-A*24:02* and *HLA-B*07:02* are independent protective HLA class I alleles for CD. In conclusion, we identified *HLA-A*02:01* and *HLA-A*02:07* as novel *HLA* class I susceptibility alleles for CD and *HLA-A*24:02* and *HLA-B*07:02* as HLA class I protective alleles for CD.

LD structure between *HLA-A*24:02, HLA-Cw*14:02* and *HLA-B*51:01*

Although we found the *HLA-A*24:02* allele to be protective and the *HLA-Cw*14:02* and *HLA-B*51:01* alleles to be associated with susceptibility for CD, these alleles constitute a common HLA haplotype in the Japanese population. Therefore, we investigated the differences in the degree of LD among these alleles between the CD and control groups. As shown in Figure 1, moderate or strong LDs were observed between *HLA-A*24:02* and *HLA-Cw*14:02* ($D' = 0.36$), between *HLA-A*24:02* and *HLA-B*51:01* ($D' = 0.51$) and between *HLA-Cw*14:02* and *HLA-B*51:01* ($D' = 0.97$) in the control group. In contrast, although the LD between *HLA-Cw*14:02* and *HLA-B*51:01* was at the same level ($D' = 0.97$) in the CD group, the LDs between *HLA-A*24:02* and *HLA-Cw*14:02* and those between *HLA-A*24:02* and *HLA-B*51:01* were weaker in the CD group ($D' = 0.13$ and 0.06, respectively) than in the controls. These results suggest that the *HLA-A*24:02-Cw*14:02-B*51:01* haplotype carries unobserved variant adjacent to *HLA-A*24:02*, which contributes to a protective effect on CD.

Table 1. Association of *HLA* class I with CD

HLA allele	*Antigen frequency (count)*		*P-value*	*OR (95% CI)*
	CD (n = 208)	*Controls (n = 384)*		
HLA-A				
*A*02*[a]	0.50 (105)	0.36 (123)	3.8×10^{-4}	1.92 (1.34–2.76)
*A*02:01*	0.29 (60)	0.20 (76)	0.016	1.64 (1.10–2.44)
*A*02:07*	0.12 (25)	0.060 (23)	0.0067	2.31 (1.26–4.24)
*A*24:02*	0.50 (104)	0.62 (239)	0.0047	0.60 (0.43–0.86)
HLA-C				
*Cw*14:02*	0.19 (39)	0.099 (38)	0.0021	2.18 (1.33–3.57)
HLA-B				
*B*07:02*	0.058 (12)	0.14 (53)	0.0041	0.38 (0.20–0.73)
*B*46:01*	0.15 (32)	0.094 (36)	0.018	1.88 (1.11–3.16)
*B*51:01*	0.20 (41)	0.13 (49)	0.033	1.70 (1.07–2.70)

Abbreviations: CA, Crohn's disease, OR, odds ratio CI, confidence interval.
[a]HLA-A*02:01, HLA-A*02:06 and HLA-A*02:07 were combined.

DISCUSSION

In our study, novel positive associations between the *HLA-A*02:01* and *HLA*-A*02:07 alleles and CD were identified. Likewise, a serologically defined *HLA-A2* allele has been previously identified as a significant susceptibility allele for CD in Caucasians.[12] Therefore, we analyzed the total frequency of all *HLA-A*02* subtypes that were increased in CD, which included *HLA-A*02:01, HLA-A*02:06* and *HLA-A*02:07* (OR = 1.92, $P = 3.8 \times 10^{-4}$) (Table 1).

Among the *HLA-A*02* alleles, *HLA-A*02:07* was more strongly associated with CD than was *HLA-A*02:01*, and *HLA-A*02:06* did not exhibit a significant positive association. To explain these differences, we compared the amino acid sequences of each *HLA-A*02* allele subtype. There is a polymorphic change from phenylalanine to tyrosine at amino acid position 9 in the *HLA-A*02:06* allele compared with the susceptibility alleles *HLA-A*02:01* and *HLA-A*02:07*, which is not associated with CD susceptibility,

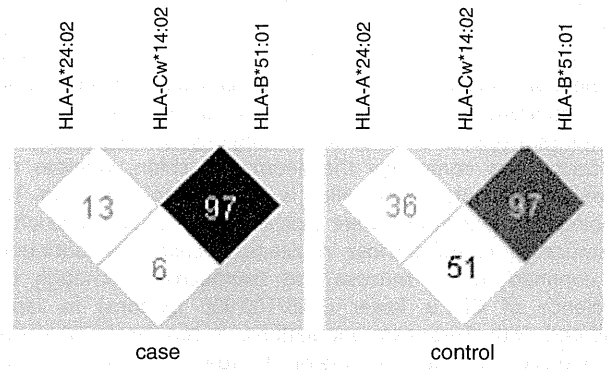

Figure 1. LD between the protective *HLA-A*24:02* allele and the susceptible *HLA-Cw*14:02* and *HLA-B*51:01* alleles for CD in cases and controls. The D′ values are indicated in each box.

Table 2. Conditional analyses of the associations between *HLA* class I and CD.

HLA	*Non-conditioned*	*Conditioned with*	
		*A*02:07*	*B*46:01*
Susceptibility allele			
*A*02:01*	0.016 (1.64, 1.10–2.44)	NA	0.012 (1.67, 1.11–2.50)
*A*02:07*	0.0067 (2.31, 1.26–4.24)	NA	0.12 (1.87, 0.85–4.11
*Cw*14:02*	0.0021 (2.18, 1.33–3.57)	0.0011 (2.29, 1.39–3.78)	0.0013 (2.26, 1.37–3.72)
*B*46:01*	0.018 (1.88, 1.11–3.16)	0.41 (1.33, 0.68–2.63	NA
*B*51:01*	0.033 (1.70, 1.07–2.70)	0.018 (1.76, 1.10–2.82)	NA
		*A*24:02*	*B*07:02*
Protective allele			
*A*24:02*	0.0047 (0.60, 0.43–0.86)	NA	0.017 (0.65, 0.46–0.93)
*B*07:02*	0.0041 (0.38, 0.20–0.73)	0.012 (0.42, 0.22–0.83)	NA

The *P*-values (OR, 95% confidence interval) obtained by conditional logistic analysis using HLA-*A*02:07* or *HLA-B*46:01* as the susceptible *HLA* allele and using *HLA-A*24:02* or *HLA-B*07:02* as protective *HLA* alleles in a dominant model are indicated.

Table 3. Polymorphic amino acid changes among *HLA-A*02* alleles.

*HLA-A*02*	*Polymorphic residue positions*				
	9	*99*	*149*	*152*	*156*
*A*02:01*	F	Y	A	V	L
*A*02:06*	**Y**	Y	A	V	L
*A*02:07*	F	**C**	A	V	L

The residues that are different among the three alleles are shown in bold face.

and a change from cysteine to tyrosine at position 99 in *HLA-A*02:01* compared with *HLA-A*02:07* (Table 3).[13]

It has been reported that the amino acids at positions 9 and 99 in HLA-A2 comprise the contact residues of the peptide binding pocket B. In these amino acid changes, the increased hydrophobicity of the residues at positions 9 and 99 has a greater effect on the susceptibility to CD (hydropathy index: phenylalanine 2.8, tyrosine −1.3, cysteine 2.5). The analysis of the amino acid changes among the *HLA-A*02:01*, *HLA-A*02:06* and *HLA-A*02:07* alleles suggests the following: (i) The phenylalanine at position 9 in *HLA-A*02* is critical to determine the susceptibility to CD. (ii) The change from cysteine to tyrosine at position 99 weakens the susceptibility to CD. In addition, *HLA-A*02:01* and *HLA-A*02:07* have been reported to bind peptides with leucine/methionine and leucine residues, respectively, at position 2, which is buried deep within peptide binding pocket B,[14,15] whereas *HLA-A*02:06* accommodates binding to valine/glutamine residues at position 2. These differences may also affect disease susceptibility to CD.

In summary, we identified two novel susceptibility HLA class I alleles, *HLA-A*02:01* and *HLA-A*02:07*, and two protective alleles, *HLA-A*24:02* and *HLA-B*07:02*, for CD in the Japanese population, although the association signals were not significant after full Bonferroni correction. It was suggested that the susceptibility conferred by the *HLA-A*02* alleles might be due to the polymorphic amino acid changes in these alleles that occur within pocket B in the antigen binding groove of the HLA-A2 molecule. All these findings may suggest a direct involvement of the HLA class I molecule in the susceptibility and resistance to CD by regulating the immune response in the gastrointestinal region.

MATERIALS AND METHODS

Subjects

Unrelated Japanese individuals with CD and healthy controls (208 and 384 individuals, respectively) were enrolled in this study. The CD diagnoses were made by gastroenterologists using conventional endoscopic, histologic and clinical criteria. Patients with indeterminate colitis were excluded in advance. Documented informed consent was obtained from each participant according to the Declaration of Helsinki. This study was also approved by the Ethics Committee at Kyushu University, Keio University and Tokyo Medical and Dental University in Japan.

Genotyping of HLA alleles

We determined the *HLA-A*, *HLA-C* and *HLA-B* genotypes (4-digit) using the Luminex assay system (Luminex Corporation, Austin, TX, USA) and HLA typing kits (Wakunaga, Hiroshima, Japan). We observed a total of 79 alleles (*HLA-A*: 21, *HLA-C*: 18 and *HLA-B*: 40 alleles). The *HLA* alleles with a frequency >0.01 (*HLA-A*: 10, *HLA-C*: 12 and *HLA-B*: 18 alleles) were subjected to further analyses.

Statistical analysis

The association between CD and the *HLA* class I antigen was assessed by logistic regression analysis with an allele dominant model adjusted for sex. For the conditional analysis, the indicated *HLA* class I allele was included in the independent variable. The *P*-values and ORs were computed using PLINK version 1.07 software (http://pngu.mgh.harvard.edu/purcell/plink/).[16] The LD among the *HLA* alleles (*HLA-A*24:02, HLA-Cw*14:02* and *HLA-B*51:01*) was calculated using Haploview version 4.2 software (http://www.broadinstitute.org/scientific-community/science/programs/medical-and-population-genetics/haploview/haploview).[17]

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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RIPK3 regulates p62–LC3 complex formation via the caspase-8-dependent cleavage of p62

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ABSTRACT

RIPK3 is a key molecule for necroptosis, initially characterized by necrotic cell death morphology and the activation of autophagy. Cell death and autophagic signaling are believed to tightly regulate each other. However, the associated recruitment of signaling proteins remains poorly understood. p62/sequestosome-1 is a selective autophagy substrate and a selective receptor for ubiquitinated proteins. In this study, we illustrated that both mouse and human RIPK3 mediate p62 cleavage and that RIPK3 interacts with p62, resulting in complex formation. In addition, RIPK3-dependent p62 cleavage is restricted by the inhibition of caspases, especially caspase-8. Moreover, overexpression of A20, a ubiquitin-editing enzyme and an inhibitor of caspase-8 activity, inhibits RIPK3-dependent p62 cleavage. To further investigate the potential role of RIPK3 in selective autophagy, we analyzed p62–LC3 complex formation, revealing that RIPK3 prevents the localization of LC3 and ubiquitinated proteins to the p62 complex. In addition, RIPK3-dependent p62–LC3 complex disruption is regulated by caspase inhibition. Taken together, these results demonstrated that RIPK3 interacts with p62 and regulates p62–LC3 complex formation. These findings suggested that RIPK3 serves as a negative regulator of selective autophagy and provides new insights into the mechanism by which RIPK3 regulates autophagic signaling.

1. Introduction

Receptor-interacting protein kinase 3 (RIP3/RIPK3) is a critical regulator of necroptosis. Necroptosis is a programmed necrotic cell death process driven by a defined molecular pathway. Necroptosis was originally observed under caspase inhibition in vitro [1,2] and confirmed in caspase-8-deficient mice [3,4]. Importantly, necroptosis can also occur in the absence of caspase inhibition in various situations [5,6], and it has an important pathophysiological role in pancreatitis [7,8], inflammatory bowel disease [9,10], and many other disorders. RIPK3 has an active kinase domain in its N-terminus that is conserved in other RIPKs and is required for necroptosis [7,11]. RIPK3 forms an intracellular complex with RIPK1 to assemble the necrosome [12]. RIPK3 also associates with the RIPK1/FADD/caspase-8 complex [7]. Recent studies have identified the mixed lineage kinase domain-like protein as an interacting partner of RIPK3 [13].

Autophagy is the common name for lysosome-based degradation of cytosolic cargos [14]. Autophagy is considered to be a nonselective, bulk process. A small portion of the cytoplasm is engulfed by an isolation membrane, which results in the formation of an autophagosome. Microtubule-associated protein 1 light chain 3 (LC3) is a marker of the autophagosome. LC3-I is subsequently conjugated with phosphatidylethanolamine (PE) to become LC3-II (LC3-PE). In contrast to the cytoplasmic localization of LC3-I, LC3-II associates with both the outer and inner membranes of the autophagosome [15]. Selective autophagy ensures the recognition and removal of various cytosolic cargos [16]. Selective autophagy is mediated by selective autophagy receptors, such as p62 (also known as sequestosome-1) and NBR1. p62 is a stress-inducible intracellular protein

Abbreviations: UBA domain, ubiquitin-associated domain; LIR, LC3-interacting region; LRS, LC3 recognition sequence; PLA, proximity ligation assay; TNT, in vitro translation.

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known to regulate various signal transduction pathways involved in cell survival and death [17]. By directly binding to LC3 via its LC3-interacting region (LIR) motif, p62 becomes a selective autophagy receptor, transporting ubiquitinated protein aggregates to the autophagosome. As p62 was identified as one of the specific substrates degraded through the autophagy–lysosomal pathway [18,19], the total cellular expression of p62 can be used to monitor autophagic flux [15]. However, recent studies indicated that p62 expression can be also regulated by transcription and protein cleavage [20,21]. In addition, p62 can suppress autophagy [22]. Although autophagy modulates the levels of p62 protein, there are potential limitations of evaluating autophagic flux by p62.

Dying cells often display an accumulation of autophagosomes and hence adopt a morphology called autophagic cell death. However, in many cases, autophagic cell death is called cell death with autophagy rather than cell death by autophagy [23]. Necroptosis is originally characterized by necrotic cell death morphology and the activation of autophagy [5], and interplay between autophagy and RIPK1-dependent necroptotic cell death has been reported [24]. However, how necroptotic and autophagic signaling proteins are recruited remains poorly understood. Given the potential importance of RIPK3 in necroptosis and the potential importance of p62 in autophagy, we investigated whether RIPK3 can regulate p62.

2. Materials and methods

2.1. Cell culture and reagents

HEK293T cells were cultured in DMEM (Sigma) with fetal bovine serum, L-glutamine, and penicillin/streptomycin at 37 °C. Mouse p62 cDNA was kindly provided by Dr. Masaaki Komatsu (Tokyo Metropolitan Institute of Medical Science). Mouse p62 cDNA was cloned into the p3XFLAG-CMV-10 expression vector (Sigma). Mouse Myc-RIPK3 and mouse Flag-A20 expression vectors were kindly provided by Dr. Averil Ma (UCSF). Human RIPK3 cDNA was amplified by RT-PCR from the total RNA of Jurkat cells and then cloned into pCMV-3tag-2C plasmids (Agilent Technologies). The pan-caspase inhibitor Z-VAD-FMK was purchased from Peptide Institute. The caspase-8 inhibitor Z-IETD-FMK was purchased from R&D SYSTEMS.

2.2. DNA and siRNA transfection

HEK293T cells were transiently transfected empty plasmid and/or expression plasmids using TransIT-LT1 (Mirus) as indicated by the supplier. OnTarget Plus SMARTpool siRNA oligonucleotides specific for human caspase-8 and nontargeting siRNA (control siRNA) were purchased from ThermoFisher Scientific. HEK293T cells seeded on 6-well plates were firstly transfected with 150 pmol of siRNA using Lipofectamine RNAiMAX (Invitrogen); after 8 h, p62 and RIPK3 expression plasmids were transfected using TransIT-LT1, according to the manufacturer's protocol.

2.3. Immunoblotting and immunoprecipitation

Cells were incubated in lysis buffer [either 20 mM HEPES (pH 7.5), 150 mM NaCl, 0.5% Triton X-100, 0.5% CHAPS, 10% glycerol, 2 mM NEM, Halt protease, and phosphatase inhibitor cocktail (Pierce) or 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.2% NP-40, 10% glycerol, 2 mM NEM, and protease inhibitors] on ice for 20 min and centrifuged at 14,000×*g* for 20 min. For Flag immunoprecipitation, cell lysates were incubated with anti-Flag M2 beads (Sigma) for 3 h at 4 °C. Samples were resolved on NuPage precast 4–12% Bis–Tris gels (Invitrogen) and transferred to a PVDF membrane. The following antibodies and reagents were used for immunoprecipitation and immunoblotting studies: anti-β-actin and anti-Flag (SIGMA); anti-LC3 (PD014, MBL), anti-A20 (5630, Cell Signaling), anti-Ub (P4D1), and anti-Myc (A-14) (Santa Cruz).

2.4. In situ proximity ligation assay (PLA)

To detect protein interactions in HEK293T cells, the Duolink PLA in situ kit (SIGMA–ALDRICH, 92101) was used, according to the manufacturer's instructions. To analyze the interaction between RIPK3 and p62 (Fig. 2), the primary antibodies were rabbit anti-c-Myc antibody (A-14, Santa Cruz) and mouse anti-Flag antibody (F3165, SIGMA). To analyze the interaction between LC3 and p62 (Fig. 4B), the primary antibodies were rabbit anti-Flag antibody (F7425, SIGMA) and mouse anti-LC3 antibody (M152-3, MBL). As a control, the primary antibody was mouse control IgG (Vector Laboratories, I-2000). Images were acquired with a confocal laser microscope (FV10i, Olympus) using a ×60 oil-immersion objective lens.

3. Results

3.1. RIPK3 mediates p62 cleavage

To investigate the potential roles of RIPK3 in regulating autophagy, we introduced Myc-mRIPK3 and Flag-p62 into HEK293T cells. HEK293T cells do not express any detectable endogenous human RIPK3 [7]. Immunoblotting analyses were performed with extracts from HEK293T cells with or without Flag-p62. A band corresponding to ~62 kDa was detected with anti-Flag antibody. This result indicated that the ~62-kDa band was the full-length p62 protein. Surprisingly, ~45-kDa and ~35-kDa bands were also observed using the same anti-Flag antibody in the presence of mouse RIPK3 (Fig. 1A). This result suggested that the ~45-kDa and ~35-kDa fragments were the products of p62 cleavage (Fig. 1A). As we performed this analysis in human HEK293T cells, we then investigated whether this p62 cleavage is specific for mouse RIPK3. We cloned human RIPK3 into an expression vector, and then human RIPK3 was transfected with Flag-p62 into HEK293T cells. Similar observations were also made using human RIPK3. Again, the ~45-kDa and ~35-kDa bands were detected in the presence of human RIPK3 (Fig. 1B). Taken together, these results suggested that RIPK3 mediates p62 cleavage in HEK293T cells.

3.2. RIPK3 forms a complex with p62

p62 regulates various signal transduction pathways and interacts with many molecules [17]. To understand how RIPK3 mediates p62 cleavage, we investigated whether RIPK3 binds to the p62 complex. We used PLA to detect whether Myc-RIPK3 interacts with Flag-p62. In this assay, a pair of oligonucleotide-labeled secondary antibodies (PLA probes) generates an individual fluorescent signal when bound to two primary antibodies in close proximity [25]. Although no PLA foci were detected in the presence of Flag-p62 or Myc-RIPK3 alone, many PLA foci were observed in the presence of both Flag-p62 and Myc-RIPK3 (Fig. 2). When we used control mouse IgG instead of mouse anti-Flag antibody, PLA foci were not observed even in the presence of both Flag-p62 and Myc-RIPK3. These data illustrated that Flag-p62 and Myc-RIPK3 interact in situ.

3.3. p62 is cleaved by caspase-8

Self-oligomerization of p62 is essential for its localization to the autophagosome formation site [26]. p62 also interacts with

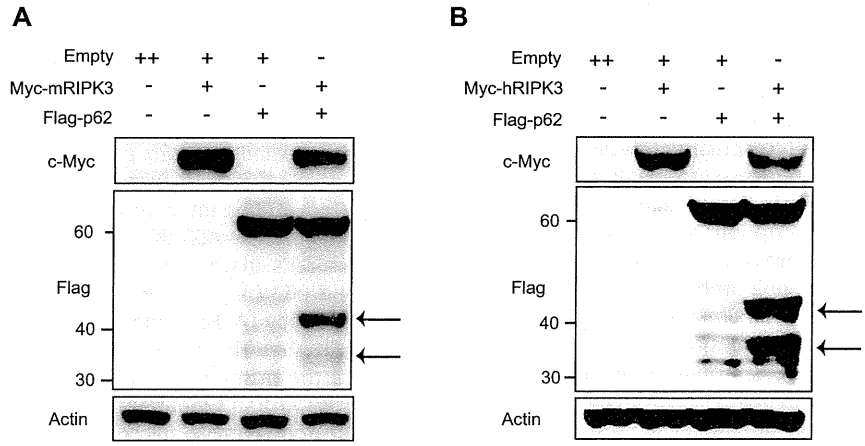

Fig. 1. Receptor-interacting protein kinase 3 (RIPK3) mediates p62 cleavage. (A) HEK293T cells were transfected with Myc-tagged mouse RIPK3 and/or Flag-tagged p62 expression plasmids. (B) HEK293T cells were transfected with Myc-tagged human RIPK3 and/or Flag-tagged p62 expression plasmids. Thirty-six hours post-transfection, cells were harvested. Flag, c-Myc, and actin antibodies were used for immunoblotting. The resultant cleavage products are indicated by arrows. The positions of the molecular mass markers are indicated in kDa at the left of the panels. Data are representative of two independent experiments.

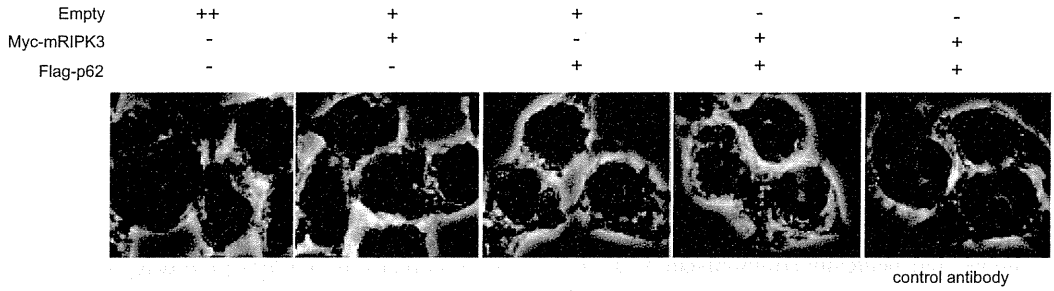

Fig. 2. Receptor-interacting protein kinase 3 (RIPK3) forms a complex with p62. HEK293T cells were transfected with Myc-tagged mouse RIPK3 and/or Flag-tagged p62 expression plasmids. Thirty-six hours post-transfection, the Duolink proximity ligation assay (PLA) demonstrated the close proximity of Flag-p62 and Myc-mouse RIPK3. Rabbit anti-c-Myc and mouse anti-Flag antibodies were used for PLA. Mouse IgG was used as a control. Data are representative of two independent experiments.

signaling molecules through several domains. The ubiquitin-associated (UBA) domain is capable of interaction with ubiquitinated proteins (Fig. 3A). p62 was identified as one of the specific substrates through the autophagy–lysosomal pathway. This degradation is mediated by an interaction with LC3 though the LIR domain [27]. Human p62 has several potential cleavage sites. TNT reaction assays revealed that human p62 is cleaved by rCaspase-6, rCaspase-8, and rCalpain [21]. A schematic representation of the domains of mouse p62 is presented in Fig. 3A. The putative cleavage site and LIR domain are highly conserved between human and mouse p62.

To study how RIPK3 mediates p62 cleavage, we first tested whether caspases induce p62 cleavage. We introduced Myc-mRIPK3 and Flag-p62 into HEK293T cells and treated the cells with caspase inhibitors. Although Myc-mRIPK3 expression might be enhanced by caspase inhibition, p62 cleavage was blocked completely by the addition of a pan-caspase inhibitor (Z-VAD-FMK) and was concentration-dependently inhibited by a caspase-8 inhibitor (Z-IETD-FMK) (Fig. 3B). These data indicated that caspases, especially caspase-8, might be involved in the regulation of p62 cleavage. However, the proper final concentration of inhibitors may vary between experiments, and high concentrations of inhibitors results in non-specific effects. Next, to test whether caspase-8 is required for p62 cleavage, we silenced caspase-8 with siRNA oligos in HEK293T cells. Caspase-8 expression was efficiently silenced in HEK293T compared with actin expression (Fig. 3C). Whereas the levels of RIPK3 in HEK293T cells might be enhanced after caspase-8 silencing, p62 cleavage was inhibited under the condition of caspase-8 deficiency (Fig. 3C). These data imply that p62 is cleaved by caspase-8. The ubiquitin-editing enzyme A20 binds to caspase-8, and overexpression of A20 inhibits caspase-8 activity in HEK293T cells [28]. To further confirm the role of caspase-8, we transfected mouse A20 together with Myc-mRIPK3 and Flag-p62. Although full-length p62 expression was not influenced by A20 expression, cleaved bands disappeared in an A20 expression-dependent manner (Fig. 3D). These data indicated that A20 may regulate RIPK3-dependent p62 cleavage though the inhibition of caspse-8 activity. Taken together, p62 is cleaved by caspase-8 under RIPK3 expression.

3.4. RIPK3 regulates complex formation between p62 and LC3

In addition to the binding capacity to LC3, p62 is also considered a receptor facilitating the delivery of ubiquitinated cargos to deliver them to the autophagosomes as a result of the presence of the UBA domain of p62 [17]. Therefore, we next examined whether p62 cleavage regulates p62 complex formation. We transfected Flag-p62 into HEK293T cells and pulled down the p62 complex with anti-Flag antibody (Fig. 4A). Flag-p62 bound to LC3-II and poly-ubiquitinated protein. Consistent with previous reports, p62 bound to LC3-II. Putative p62 cleavage sites are located upstream of the LC3 binding site and ubiquitin binding site. Based on predictive algorithms, the ~45-kDa fragments of p62 lacked ubiquitin binding sites, and the ~35-kDa fragments lacked ubiquitin binding sites and an LC-3 binding site. When we transfected Myc-mRIPK3 together with Flag-p62, we confirmed Myc-mRIPK3 in association with Flag-p62 by both the PLA and immunoprecipitation. Moreover, p62–LC3 and p62–polyubiquitin complex formation was prevented by the presence of RIPK3. These data illustrated that RIPK3 regulates p62 complex formation by p62 cleavage.

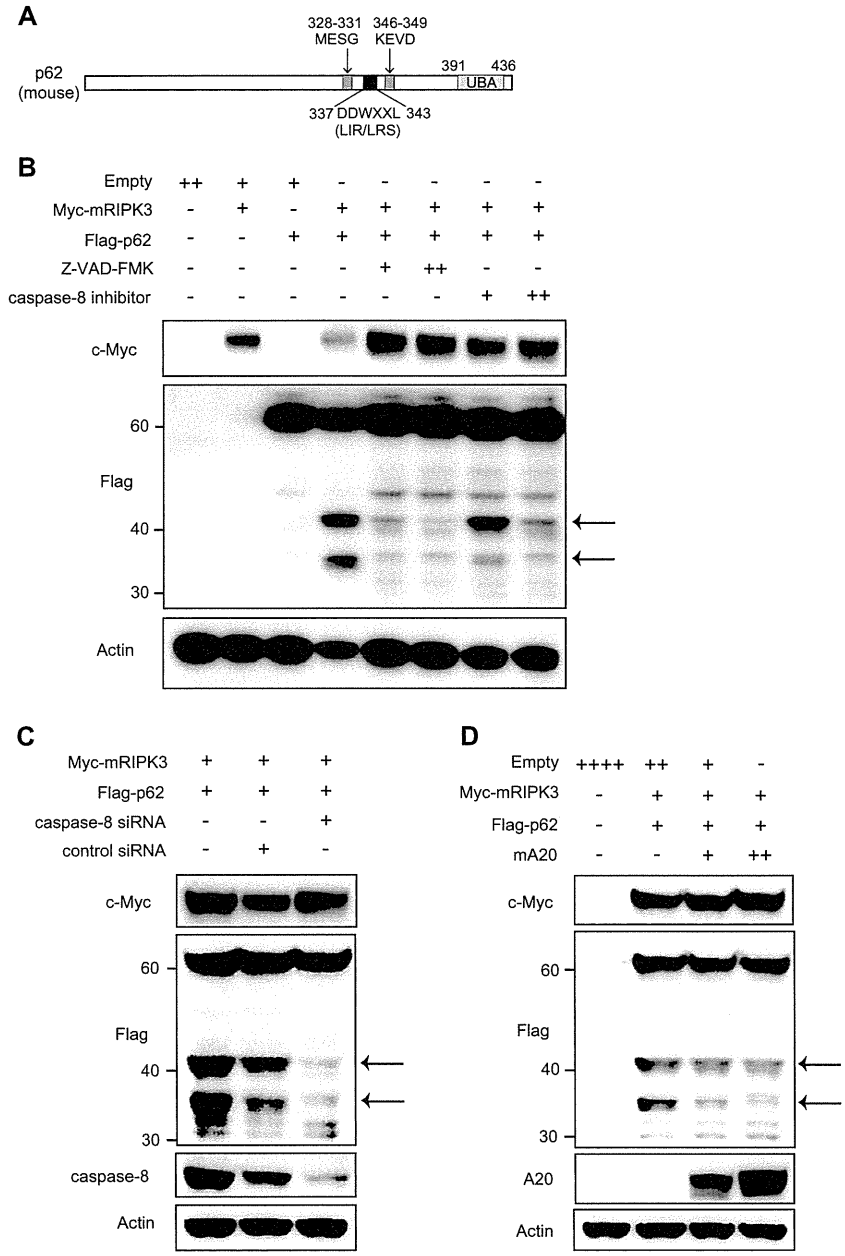

Fig. 3. p62 is cleaved by caspase-8. (A) Domain structure of mouse p62. Microtubule-associated protein 1 light chain 3 (LC3) recognizes p62 through the LC3-interacting region (LIR)/LC3 recognition sequence (LRS) domain. p62 interacts with ubiquitinated proteins via the ubiquitin-associated domain. Potential caspase-8 cleavage sites in mouse p62 were mapped to its C-terminus. Cleavage sites are based on predictive algorithms [21]. (B) HEK293T cells were transfected with Myc-tagged mouse receptor-interacting protein kinase 3 (RIPK3) and/or Flag-tagged p62 expression plasmids. Cells were treated with Z-VAD-FMK (20 or 50 nM) or caspase-8 inhibitor (10 or 50 nM) for 24 h. (C) HEK293T cells were transfected with a nontargeting control siRNA or siRNAs against human caspase-8. Eight hours later, Myc-tagged mouse RIPK3 and/or Flag-tagged p62 expression plasmids were transfected. After 36 h, cells were lysed and analyzed by immunoblotting. (D) HEK293T cells were transfected with Myc-tagged mouse RIPK3, Flag-tagged p62, and/or mouse A20 expression plasmids. Thirty-six hours post-transfection, cells were harvested. All data are representative of three independent experiments.

To confirm these observations in situ, we perform PLA using Flag and LC-3 antibody. We detected the Flag-p62–LC3 complex in situ in HEK293T cells. We observed decreased numbers of PLA foci in the presence of RIPK3 (Fig. 4B). This result is consistent with a previous report demonstrating that the colocalization of LC3 with the p62 mutant (lacking both LIR and the UBA domain) was profoundly inhibited [26]. In addition, RIPK3-dependent disruption of p62–LC3 complex is restored by caspase inhibition. These data indicated that p62 cleavage regulates p62–LC3 complex formation. Taken together, RIPK3 regulates the association of p62 with LC3 through caspase-8 activity.

4. Discussion

We have identified a unique mechanism by which RIPK3 regulates p62–LC3 complex formation. Our results indicated that RIPK3 forms a complex with p62 and mediates p62 cleavage by caspase-8. These findings provide new insights into the mechanism by which necroptosis signaling regulates autophagic signaling.

To investigate the RIPK3-dependent mechanism, we performed co-transfection assays in human HEK293T cells. HEK293T can sufficiently mimic RIPK3-deficient cells with high transient transfection efficiency [8,29]. As the p62 amino acid sequence is highly

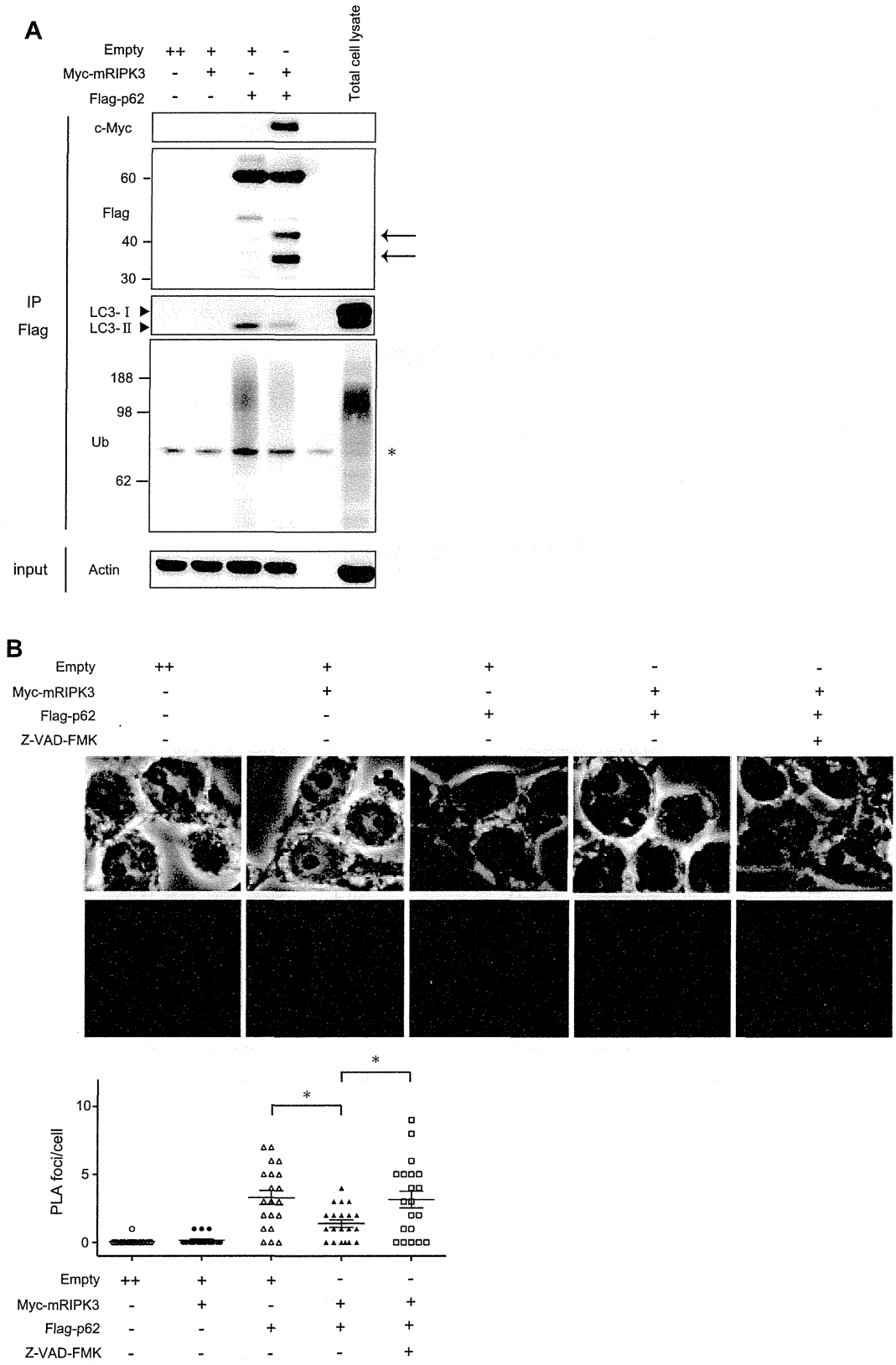

Fig. 4. Receptor-interacting protein kinase 3 (RIPK3) regulates the association of p62 with microtubule-associated protein 1 light chain 3 (LC3). (A) RIPK3 releases LC3 from p62. HEK293T cells were transiently cotransfected with mouse RIPK3 and/or p62 expression plasmids. Thirty-six hours post-transfection, protein extracts were immunoprecipitated (IP) with Flag antibody and immunoblotted for the indicated proteins. The total cell lysate of HEK293T cells was used as a positive control for immunoblotting. *: indicates a nonspecific band. (B) HEK293T cells were transfected with Myc-tagged mouse RIPK3 and/or Flag-tagged p62 expression plasmids. Thirty-six hours post-transfection, the Duolink proximity ligation assay (PLA) demonstrated the close proximity of Flag-p62 and LC-3. Rabbit anti-Flag and mouse anti-LC-3 antibodies were used for PLA. Mouse IgG was used as a control. Cells were treated with Z-VAD-FMK (50 nM) for 24 h. The numbers of PLA foci per cell are shown ($n = 20$ per group). The student's unpaired t-test was performed for the statistical analysis. *$P \leqslant 0.05$. Data are representative of two independent experiments.

conserved between humans and mice, we used a mouse p62 expression vector. Thus, we could detect RIPK3-dependent p62 cleavage.

Human RIPK3 is cleaved at Asp328 by caspase-8 in HeLa or 293T cells under stimulation [29]. In contrast to the cleavage of p62, we could not detect obvious cleavage of RIPK3 in our study. A potential explanation is that we used HEK293T cells without stimulation. However, overexpression of RIPK3 mediates caspase-8-dependent p62 cleavage. Because a previous paper revealed that p62 associates with caspase-8 [28], RIPK3 may regulate p62–caspase-8 complex formation. In addition, there were some weak bands other than the ~45-kDa and ~35-kDa fragments (Figs. 1 and 3). These bands might be cleaved by other enzymes, such as caspase-6 [21]. Further investigation is required to clarify the mechanisms by which RIPK3 regulates p62 cleavage.

Although cleaved fragments of p62 disappeared upon caspase inhibition, full-length p62 expression was not obviously affected (Fig. 3B–D). However, we revealed that RIPK3-dependent disruption of the p62–LC3 complex was regulated by caspase inhibition. One potential reason is that cleaved fragments of p62 may prevent full-length p62 self-oligomerization [26]. In contrast to p62, Myc-RIPK3 protein expression may be enhanced by caspase inhibition in our systems (Fig. 3B–D). These results are consistent with a previous study reporting that RIPK3 protein was significantly overexpressed in the small intestine in caspase-8-deficient mice compared with the findings in the control littermate mice [9].

The total cellular expression levels of p62 can be used to monitor autophagic flux [15]. However, we demonstrated that p62 expression is regulated by some types of cleavage under RIPK3 expression. This may be an important mechanism of selective autophagy to control protein degradation during necroptosis.

Our data illustrated that RIPK3 interferes with p62–LC3 complex formation in the absence of Z-VAD treatment. These data indicated that RIPK3 constitutively attenuates selective autophagy, including the degradation of aggresomes, mitochondria, and invading bacteria [30]. When necroptosis is triggered by Z-VAD, death receptors, and other signals, RIPK3 induces downstream death signaling and enhances selective autophagy. On the contrary, a recent paper uncovered that p62 can suppress autophagy via mTORC1 activation [17,22,31]. These findings indicated that RIPK3 may regulate both selective autophagy and the induction of autophagy via the caspse-8-dependent cleavage of p62 under some conditions. However, to confirm this hypothesis, we need to analyze RIPK3 kinase-dead mutants under necroptotic stimulation. Because necroptosis can also occur in the absence of caspase inhibition, we should analyze RIPK3-dependent disruption of the p62–LC3 complex under pathophysiological conditions [32].

In conclusion, we demonstrated the functions of RIPK3 in regulating p62 cleavage by casapse-8. These functions are critical for p62–LC3 complex formation. In addition to a new molecular mechanism of p62 regulation, these studies provide critical insights into the regulation of necroptosis and autophagy.

Conflict of interest

The authors declare that they have no conflicts of interest.

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