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研究課題名（和文）Functional investigation of Cables2, a novel transcription cofactor regulating Nanog expression through Smad2 activation, during germ cell development

研究課題名（英文）Functional investigation of Cables2, a novel transcription cofactor regulating Nanog expression through Smad2 activation, during germ cell development

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交付決定額（研究期間全体）：（直接経費）　　3,200,000円

研究成果の概要（和文）：Cablesファミリーに属するCDK5 and Abl enzyme substrate 2 (Cables2)は、マウス胚および生殖器官で遍在的に発現する。本研究では、in vivoでのCables2の機能解明のため、マウスを用いた表現型解析を行った。
遺伝子改変マウスの解析によりCables2座位全体がマウス初期発生に必須であった。トランスクリプトームの解析では、Cables2 がオス生殖器発生に重要で、Wntやp53のシグナル伝達経路に関与していることが明らかになった。 また、Cables2座位へのレポーターのノックインに成功し、精巣と卵巣での特異的発現を確認した。

研究成果の学術的意義や社会的意義
This study firstly report about the essential of Cables2 locus in mouse development. Cables2 is clarified to be expressed in various mouse tissues including testis, ovary, brain, etc. In addition, this study focus attention on the need to validate target knock-out genes in lethal phenotypes.

研究成果の概要（英文）：CDK5 and Abl enzyme substrate 2 (Cables2), a member of the Cables family, is ubiquitously expressed in mouse embryonic and reproductive organs at the RNA level, suggesting the existence and contribution of Cables2 in embryonic and germ cell development. However, in vivo function of Cables2 remains unclear. Therefore, in this study, we aimed to explore the role of Cables2 using mouse models.
Using genetically modified mouse models, we found that the entire Cables2 locus is essential for early mouse embryogenesis. Transcriptome profiling comparison revealed that Cables2 indeed involved in male gonad development and fundamental signaling pathways such as Wnt and P53. In addition, we successfully generated a Cables2 knock-in reporter model in which Cables2 is demonstrated to be expressed in mouse testis and ovary, the main reproductive organs. Generally, this study highlights the sophisticated role of Cables2 in mouse development.

研究分野：42040

キーワード：Cables2　embryogenesis

１．研究開始当初の背景

CDK5 and Abl enzyme substrate 2 (Cables2), a member of the Cables family that has a C-terminal cyclin box-like domain, is ubiquitously expressed in mouse embryos, from embryonic stem cells (ESCs) to prenatal embryos and adult tissues. In humans, *Cables2* is also expressed in various tissues including the testis, ovary, oviduct, uterus, etc. with the highest RNA level in the human testis *(www.proteinatlas.org)*. The conventional *Cables2* KO embryos, in which the entire Cables2 locus was deleted, are arrested for the normal development from the gastrulation, embryonic day 6.5 (E6.5).

Our preliminary *in vitro* data showed that *Nanog* and Smad2 activity were decreased in *Cables2*-deficient epiblast-like cells (EpiLCs), suggesting that Cables2 is a novel transcription cofactor of Nodal/Smad2 signaling pathways in regulating Nanog expression in gastrulation. Therefore, we initially planned to apply Cre/loxP system to analyze the germ cell-specific function of *Cables2*.

２．研究の目的

In this study, we aimed to explore the temporal function of *Cables2* in mouse development.

３．研究の方法

1. Generation of *Cables2-3×FLAG-2A-tdTomato* (*Cables2*^{Tom}) mouse to confirm the expression of Cables2 *in vivo*. In this model, the C-terminal of *Cables2* was knocked in with 3xFLAG and 2A-mediated tdTomato.
2. Generation of *Cables2* cKO mouse: Using the CRISPR/Cas9 system, the floxed *Cables2* mouse with loxP sites flanking exon1 was successfully generated (**Fig.2**). The floxed *Cables2* mice were mated with *Nanos3-Cre* and *Gdf9-Cre* mouse lines where *Cre* is specifically expressed in male and female germ cells, respectively.
3. RNA sequencing analysis: The Enrichr program was used for GO terms and KEGG pathway enrichment analyses of differentially expressed genes with at least twofold change and FDR < 0.05.

４．研究成果

1. Expression pattern of Cables2 in mouse reproductive organs

Instead of using cell lines, the bicistronic *Cables2* knock-in reporter mice that expressed Cables2 tagged with a fluorescent reporter were

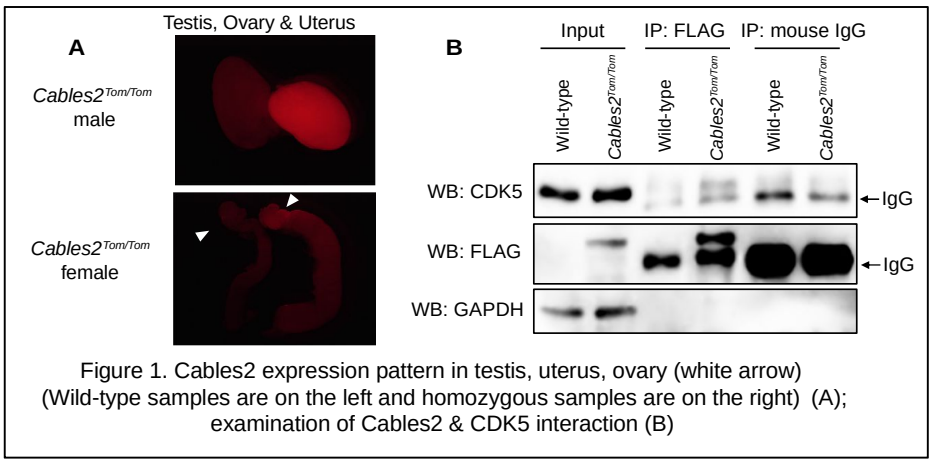

Figure 1. Cables2 expression pattern in testis, uterus, ovary (white arrow) (Wild-type samples are on the left and homozygous samples are on the right) (A); examination of Cables2 & CDK5 interaction (B)

generated and utilized for *in vivo* analysis, abbreviated as *Cables2*^{Tom}. As result shown in **Figure 1A**, the reporter mice of *Cables2* showed the high Cables2 expression in testis and ovary. Moreover, Cables2 is widely expressed in various mouse tissues including brain, intestine, kidney, etc. Further immunoprecipitation analysis using testis of Cables2 reporter mice revealed interaction of Cables2 with CDK5, a well-known interaction factor of Cables (**Fig.1B**). Due to lacking commercially valuable Cables2 antibody, this knock-in mouse model will enable the comprehensive analysis of *in vivo* Cables2 function in both male and female reproductive organs. However, we could not observe the significant expression of reporter fluorescence in primordial germ cells and embryos using this knock-in mouse.

2. Generation of *Cables2* conditional KO mice and finding the discrepant phenotypes in *Cables2* deletion models

The conventional *Cables2* KO model, in which the entire *Cables2* locus was deleted (**Fig.2**) showed the lethal phenotype. Homozygous embryos are arrested from the onset of gastrulation at embryonic day 6.5 (E6.5). Therefore, the floxed *Cables2* mouse with loxP sites flanking exon1, which is considered a critical exon, was generated and mated with *Nanos3*-Cre and *Gdf9*-Cre mice to examine the function of *Cables2* in germ cells. However, *Cables2* conditional KO mice in both lines are normal fertility with no obvious abnormal phenotype.

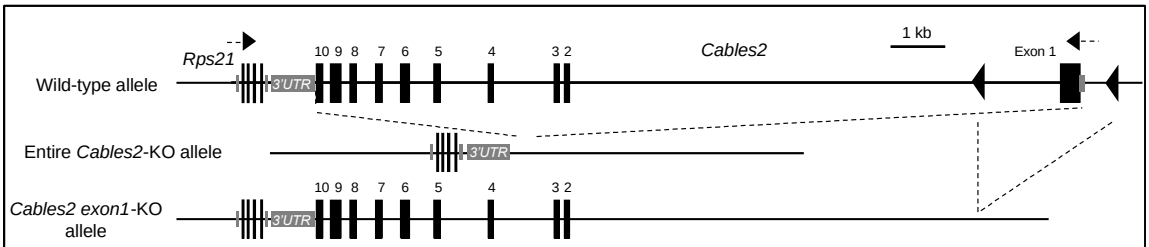

Figure 2. Gene construction of conventional targeting entire *Cables2* locus deletion and *Cables2* exon1 deletion

To further validate the efficiency of exon1-floxed construction and re-confirm the lethal phenotype, *Cables2* exon1 deletion mice were exclusively intercrossed and propagated. Unexpectedly, viable and fertile homozygous *Cables2* exon1 deletion mice were obtained, which is contrary to the entire locus *Cables2* phenotype. Thus, our conditional KO model seems insufficiency to reflect the function of *Cables*, unfortunately. This surprising result also indicates an inconsistent function of *Cables2* in embryogenesis.

3. The transcriptome profiling comparison of entire *Cables2* locus deletion embryos

As the initial *in vitro* data suggested that Cables2 may play an important role in the activity of Nodal/Smad2 signaling pathways during germ cell development. However, the *in vivo* results afterward did not strongly support our hypothesis about the specific function of Cables2. On the other hand, we found that targeted disruption of the entire *Cables2* locus caused growth retardation and enhanced apoptosis at the gastrulation stage and then induced embryonic lethality in mice. Therefore, we performed RNA-seq with conventional KO embryo E7.5 samples to examine global gene changes. Interestingly, the GO terms related to “gonad development”, “male gonad development”, and “development of primary male sexual characteristics”(**Fig.3A**). Moreover, the enriched KEGG pathway included “Wnt signaling pathway”, “PI3K-Akt signaling pathway” and “p53 signaling pathway” (**Fig.3B**). Comparative transcriptome analysis also

revealed that disruption of *Cables2* affected the gene expression of *Rps21*, a gene located next to the exon 10 of the *Cables2* locus in the opposite orientation. Generally, Cables2 is involved in male gonad development and has sophisticated interaction with adjacent gene *Rps21*, Wnt/b-catenin, and P53 signaling pathways. These findings would contribute to the comprehensive understanding of Cables2 function.

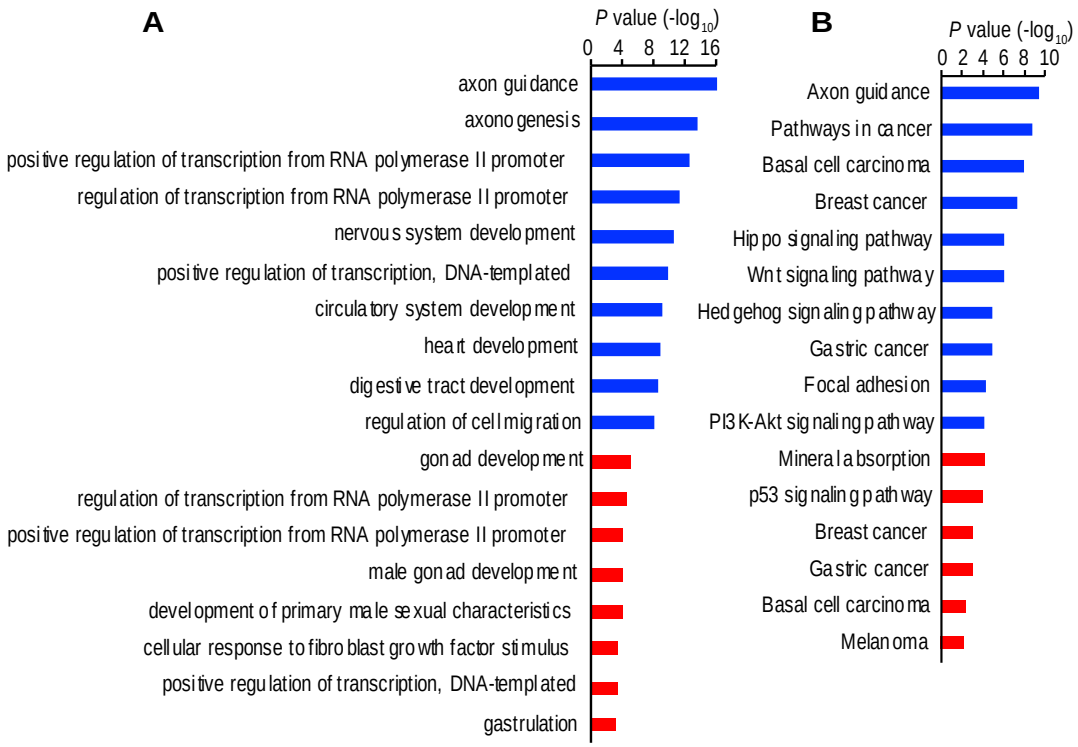

Figure 3. Transcriptome profiling analysis of entire *Cables2* deletion embryos Different expression genes at E7.5 enriched in GO Biological process (A) and KEGG pathway (B) ($p<0.001$) with downregulation in blue bars and upregulation in red bars.

５．主な発表論文等

〔雑誌論文〕　計2件（うち査読付論文　2件／うち国際共著　2件／うちオープンアクセス　2件）

１．著者名 Hasan Ammar Shaker Hamed、Dinh Tra Thi Huong、Le Hoai Thu、Mizuno-Iijima Saori、Daitoku Yoko、Ishida Miyuki、Tanimoto Yoko、Kato Kanako、Yoshiki Atsushi、Murata Kazuya、Mizuno Seiya、Sugiyama Fumihiro	４．巻 70
２．論文標題 Characterization of a bicistronic knock-in reporter mouse model for investigating the role of CABLES2 in vivo	５．発行年 2021年
３．雑誌名 Experimental Animals	６．最初と最後の頁 22～30
掲載論文のDOI（デジタルオブジェクト識別子） 10.1538/expanim.20-0063	査読の有無 有
オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 該当する

１．著者名 Dinh Tra Thi Huong、Iseki Hiroyoshi、Mizuno Seiya、Iijima-Mizuno Saori、Tanimoto Yoko、..., Murata Kazuya、Muratani Masafumi、Ema Masatsugu、Kim Jun-Dal、Ishida Junji、Fukamizu Akiyoshi、Kato Mitsuyasu、Takahashi Satoru、Yagami Ken-ichi、Wilson Valerie、Arkell Ruth M、Sugiyama Fumihiro	４．巻 10
２．論文標題 Disruption of entire Cables2 locus leads to embryonic lethality by diminished Rps21 gene expression and enhanced p53 pathway	５．発行年 2021年
３．雑誌名 eLife	６．最初と最後の頁 e50346
掲載論文のDOI（デジタルオブジェクト識別子） 10.7554/eLife.50346	査読の有無 有
オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 該当する

〔学会発表〕　計3件（うち招待講演　1件／うち国際学会　1件）

１．発表者名 Tra Thi Huong Dinh, Hiroyoshi Iseki, Seiya Mizuno, Saori Iijima-Mizuno, Yoko Tanimoto, Yoko Daitoku, Kanako Kato, Masatsugu Ema, Akiyoshi Fukamizu, Mitsuyasu Kato, Satoru Takahashi, Ken-ichi Yagami, Fumihiro Sugiyama
２．発表標題 Cables2 regulates mouse gastrulation by stimulating Wnt/beta-catenin signalling pathway
３．学会等名 The 2019 International Symposium of Korean Association for Laboratory Animal Sciences (KALAS)（招待講演）（国際学会）
４．発表年 2019年

１．発表者名 Tra Thi Huong Dinh, Hiroyoshi Iseki, Seiya Mizuno, Satoru Takahashi, and Fumihiro Sugiyama.
２．発表標題 Disruption of entire Cables2 locus leads to embryonic lethality by diminished Rps21 gene expression and enhanced p53 pathway.
３．学会等名 The 94th Annual Meeting of the Japanese Biochemical Society (JBS), 2T14a-06 (P-610), Yokohama, Japan (Nov., 2021)
４．発表年 2021年

１．発表者名
Tra Thi Huong Dinh, Hiroyoshi Iseki, Seiya Mizuno, Masatsugu Ema, Akiyoshi Fukamizu, Satoru Takahashi, Ken-ichi Yagami and Fumihiro Sugiyama.

２．発表標題
The essential of entire Cables2 locus for early embryonic development in mice.

３．学会等名
The 44th Annual Meeting of the Molecular Biology Society of Japan (MBSJ), 3LBA-051, Yokohama, Japan (Dec., 2021)

４．発表年
2021年

〔図書〕　計0件

〔産業財産権〕

〔その他〕

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６．研究組織

	氏名 （ローマ字氏名） （研究者番号）	所属研究機関・部局・職 （機関番号）	備考

７．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計0件

８．本研究に関連して実施した国際共同研究の実施状況

共同研究相手国	相手方研究機関