

#### 4. Discussion

For investigation of the roles played by specific mAChR subtypes in the regulation of immune function, the use of mice with a specific mAChR subtype gene deficiency has advantages over classical pharmacological approaches in that the deficit is highly specific, and the inhibition is complete. Nevertheless, the results obtained with mice deficient in a specific mAChR subtype must be viewed with some caution, as the affected mAChR subtype will be absent from cells throughout the body, and indirect effects on immune system function mediated by the nervous and/or endocrine systems cannot be ruled out. That said, the results of the present study demonstrate for the first time that a lymphocytic cholinergic system acts *via* mAChR-mediated pathways to regulate antibody production. Our finding that anti-OVA-specific IgG<sub>1</sub> production and antigen-specific IL-6 secretion observed 1 wk after immunization was lower in M<sub>1</sub>/M<sub>5</sub> KO mice than in wild-type mice suggests M<sub>1</sub> and/or M<sub>5</sub> mAChR signaling may modulate antibody class switching from IgM to IgG<sub>1</sub> *via* an effect on cytokine (*e.g.*, IL-6) production. That there was no change in anti-OVA-specific serum IgM indicates these receptors do not mediate effects on the initial generation of the antibody response. The genetic switch from IgM to IgG production can occur *via* two distinct pathways. One involves IFN- $\gamma$  (a Th1 type cytokine) stimulating IgM-secreting cells to switch to IgG<sub>2a</sub>-secreting cells (Coffman et al., 1993), while the other involves IL-4 or IL-6 (two Th2 type cytokines) stimulating IgM-secreting cells to switch to IgG<sub>1</sub>-secreting cells (Coffman et al., 1993; Van Ommen et al., 1994). When we compared antigen-specific cytokine production in OVA-activated spleen cells, we did not observe a significant change in IFN- $\gamma$  production, but we did find that IL-6 production was significantly diminished in M<sub>1</sub>/M<sub>5</sub> KO mice. In addition, there was a significant correlation between serum levels of anti-OVA-specific IgG<sub>1</sub> and antigen-specific IL-6 production (Table 1), which is consistent with IL-6 being involved in regulating antigen-specific IgG<sub>1</sub> production. However, the precise mechanisms by which stimulation of M<sub>1</sub> and/or M<sub>5</sub> mAChRs mediates Th2 type cytokine production in immune cells remains to be determined. We did not measure IgG<sub>2a</sub> in the present study because it has been established that C57BL/6 mice are genetically incapable of producing significant amounts of the Th1-associated IgG<sub>2a</sub> (Martin et al., 1998).

While we observed that both total serum IgG<sub>1</sub> and serum anti-OVA-specific IgG<sub>1</sub> were significantly lower in M<sub>1</sub>/M<sub>5</sub> KO mice, we found no significant differences in total serum IgM or serum anti-OVA-specific IgM between the two genotypes (Figs. 1 and 2). This suggests that the immune system defect in M<sub>1</sub>/M<sub>5</sub> KO mice is limited to the IgG<sub>1</sub> response and that there is not a general retardation of immune system development in these mice.

We found that 1 wk after the immunization with OVA total serum IgG<sub>1</sub> and IgM levels were more than 5000 times higher than antigen-specific IgG<sub>1</sub> and IgM, respectively. The significant reduction of total serum IgG<sub>1</sub> seen in M<sub>1</sub>/M<sub>5</sub> KO mice suggests that IgG<sub>1</sub> synthesis in B cells is constitutively suppressed in M<sub>1</sub>/M<sub>5</sub> KO mice. Because IgM antibodies have low affinities for antigens, antibody class switching to produce

high affinity IgG antibodies is very important for the maintenance of immunocompetence. The observed reductions in serum levels of total and antigen-specific IgG<sub>1</sub> in M<sub>1</sub>/M<sub>5</sub> KO mice suggest the possibility that prolonged administration of antimuscarinic drugs to elderly patients with urinary incontinence or chronic obstructive pulmonary disease could bring on a condition in which the patient was immunocompromised.

The nonspecific mAChR agonist oxotremorine (Oxo)-M induces Ca<sup>2+</sup>-signaling and up-regulation of *c-fos* expression in both T and B cells, further suggesting the involvement of mAChRs in the regulation of lymphocyte function (Fujii and Kawashima, 2000a,b). Because no agonist or antagonist selective for only a single mAChR subtype is currently available, it is impossible to directly determine which mAChR subtype is responsible for the observations summarized above using pharmacological techniques. However, 4-DAMP, an M<sub>1</sub>, M<sub>3</sub>, M<sub>4</sub> and M<sub>5</sub> mAChR antagonist; YM905, an M<sub>1</sub> and M<sub>3</sub> mAChR antagonist; and atropine, a nonselective mAChR antagonist, all reversed Oxo-M-induced Ca<sup>2+</sup>-signaling and up-regulation of *c-fos* expression at lower concentrations than did AF-DX 116, an M<sub>2</sub> and M<sub>4</sub> mAChR antagonist (Fujii and Kawashima, 2000a,b, 2001b). Collectively, these findings suggest that ACh induces Ca<sup>2+</sup>-signaling in lymphocytes *via* M<sub>1</sub>, M<sub>3</sub> and/or M<sub>5</sub> mAChRs, leading to IP<sub>3</sub>-mediated up-regulation of *c-fos* expression, which in turn leads to activation of DNA and RNA synthesis and cell proliferation (Fujii and Kawashima, 2000a,b, 2001b; Kawashima and Fujii, 2000). The results of the present study demonstrate concretely for the first time that M<sub>1</sub> and/or M<sub>5</sub> mAChRs play a key role in the regulation of immune cell activity.

Szelenyi et al. (1982) detected AChE activity in human peripheral blood T cells, where the activity was augmented by PHA. In addition, Ando et al. (1999) detected expression of three distinct mRNAs encoding the hydrophilic, phosphoinositide-linked and readthrough forms of AChE in human MNLs and in human leukemic T and B cell lines. Similarly, Tayebati et al. (2002) detected AChE expression in both T and B cells using Western and immunocytochemical analyses. In our recent studies, we detected AChE mRNA expression in splenic MNLs, dendritic cells and macrophages from C57BL/6Jcl mice (Kawashima et al., 2007). The down-regulation of AChE mRNA expression we saw in spleen cells from M<sub>1</sub>/M<sub>5</sub>KO C57BL/6Jcl mice (Fig. 4) was also seen in a preliminary study using M<sub>5</sub>KO DBA/2Jcl mice (unpublished data). This suggests that M<sub>5</sub> mAChR signaling in some way modulates AChE transcription, though the precise mechanism remains unclear.

At neuromuscular and neuroeffector junctions, AChE rapidly hydrolyzes ACh, terminating its activity. A substantial prolongation in the decay time constant of focally recorded miniature endplate currents has been observed in AChE KO mice, as compared with wild-type mice (5.4±0.3 ms vs. 1.04±0.06 ms) (Girard et al., 2007), which is indicative of the role played by AChE at the neuromuscular junction. On the other hand, the importance to immune system function of the small but significant reduction of AChE expression observed in M<sub>1</sub>/M<sub>5</sub> KO mice remains unclear. One would predict, however, that reduced expression of AChE mRNA in M<sub>1</sub>/M<sub>5</sub> KO mice would lead to enhanced cholinergic transmission *via* M<sub>2</sub>, M<sub>3</sub>

and M<sub>4</sub> mAChRs and/or nAChRs, reflecting the attenuated ACh degradation. In addition, Skok et al. (2005) showed that IgG immune responses were stronger in nAChR 4- or 2-deficient mice than in wild-type mice and suggested that the AChR 4 and 2 subunits were involved in down-regulating IgG production. We therefore cannot rule out the possibility that the reduced antigen-specific IgG<sub>1</sub> production observed in M<sub>1</sub>/M<sub>5</sub> KO mice in the present study is attributable in part to an indirect facilitation of cholinergic transmission *via* nAChR.

In an earlier study, we showed that stimulation of splenic MNLs with ConA induced ChAT mRNA expression, which is consistent with the idea that T cell activation leads to enhanced cholinergic transmission (Kawashima et al., 2007). On the other hand, no ChAT transcription was detected under the resting conditions. This is most likely because splenic MNLs include only a small number of T cells (about 15%) and an even smaller number of CD4<sup>+</sup> T cells (6%), which play the major role in ChAT-catalyzed ACh synthesis (Fujii et al., 2003a; Kawashima et al., 2007). We also did not detect expression ChAT mRNA in the present study, even after the stimulation with OVA. This suggests that with the spleen cell samples tested, the number of T cell clones specifically reactive to OVA was too small for expression of ChAT mRNA to be detected by RT-PCR, despite the presence of large numbers of CD4<sup>+</sup> T cells (30%).

In summary, we found that total serum IgG<sub>1</sub> levels and levels of anti-OVA-specific IgG<sub>1</sub> and IL-6 production were all diminished in M<sub>1</sub>/M<sub>5</sub> KO mice 1 wk after immunization with OVA, though there was no change in serum levels of antigen-specific IgM. This suggests the involvement of M<sub>1</sub> and/or M<sub>5</sub> mAChRs in the regulation of immune function, at least in part through altered production of the Th2 type cytokine IL-6, leading to modulation of antibody class switching from IgM to IgG<sub>1</sub>.

## References

- Alexander, S.P.H., Peters, J.A., 1999. Tips receptor and ion channel nomenclature (10th ed.). Trends Pharmacol. Sci. 20, 6–8 (Suppl.).
- Ando, T., Fujii, T., Kawashima, K., 1999. Expression of three acetylcholinesterase mRNAs in human lymphocytes. Japan. J. Pharmacol. 79 (Suppl. I), 289.
- Bonner, T.I., Buckley, N.J., Young, A.C., Brann, M.R., 1987. Identification of a family of muscarinic acetylcholine receptor genes. Science 237, 527–532.
- Bonner, T.I., Young, A.C., Brann, M.R., Buckley, N.J., 1988. Cloning and expression of the human and rat m5 muscarinic receptor genes. Neuron 1, 403–410.
- Coffman, R.L., Leberman, D.A., Rothman, P., 1993. Mechanisms and regulation of immunoglobulin isotype switching. Adv. Immunol. 54, 229–270.
- Fujii, T., Kawashima, K., 2000a. Calcium oscillation is induced by muscarinic acetylcholine receptor stimulation in human leukemic T- and B-cell lines. Naumyn-Schmiedeberg's Arch. Pharmacol. 362, 14–21.
- Fujii, T., Kawashima, K., 2000b. Calcium signaling and *c-fos* gene expression via M<sub>3</sub> muscarinic acetylcholine receptors in human T- and B-cells. Japan. J. Pharmacol. 84, 124–132.
- Fujii, T., Kawashima, K., 2001a. The non-neuronal cholinergic system: an independent non-neuronal cholinergic system in lymphocytes. Japan. J. Pharmacol. 85, 11–15.
- Fujii, T., Kawashima, K., 2001b. YM905, a novel M<sub>3</sub> antagonist, inhibits Ca<sup>2+</sup> signaling and *c-fos* gene expression mediated via muscarinic receptors in human T cells. Gen. Pharmacol. 35, 71–75.
- Fujii, T., Masuyama, K., Kawashima, K., 2006. Simvastatin regulates non-neuronal cholinergic activity in T lymphocytes via CD11a-mediated pathways. J. Neuroimmunol. 179, 101–107.
- Fujii, T., Tsuchiya, T., Yamada, S., Fujimoto, K., Suzuki, T., Kasahara, T., Kawashima, K., 1996. Localization and synthesis of acetylcholine in human leukemic T-cell lines. J. Neurosci. Res. 44, 66–72.
- Fujii, T., Ushiyama, N., Hosonuma, K., Suenaga, A., Kawashima, K., 2002. Effects of human antithymocyte globulin on acetylcholine synthesis, its release and choline acetyltransferase transcription in a human leukemic T-cell line. J. Neuroimmunol. 128, 1–8.
- Fujii, T., Watanabe, Y., Inoue, T., Kawashima, K., 2003a. Up-regulation of mRNA encoding the M<sub>5</sub> muscarinic acetylcholine receptor in human T- and B-lymphocytes during immunological responses. Neurochem. Res. 28, 423–429.
- Fujii, T., Watanabe, Y., Fujimoto, K., Kawashima, K., 2003b. Expression of acetylcholine in lymphocytes and modulation of an independent lymphocytic cholinergic activity by immunological stimulation. Biog. Amines 17, 373–386.
- Fujii, T., Yamada, S., Misawa, H., Tajima, S., Fujimoto, K., Suzuki, T., Kawashima, K., 1995. Expression of choline acetyltransferase mRNA and protein in T-lymphocytes. Proc. Jpn. Acad. 71B, 231–235.
- Fujii, T., Yamada, S., Watanabe, Y., Misawa, H., Tajima, S., Fujimoto, K., Kasahara, T., Kawashima, K., 1998. Induction of choline acetyltransferase mRNA in human mononuclear leukocytes stimulated by phytohemagglutinin, a T-cell activator. J. Neuroimmunol. 82, 101–107.
- Girard, E., Bernard, V., Minic, J., Chatonnet, A., Krejci, E., Molg6, J., 2007. Butyrylcholinesterase and the control of synaptic responses in acetylcholinesterase knockout mice. Life Sci. 80, 2380–2385.
- Hulme, E.C., Birdsall, N.J.M., Buckley, N.J., 1990. Muscarinic receptor subtypes. Ann. Rev. Pharmacol. Toxicol. 30, 633–673.
- Kawashima, K., Fujii, T., 2000. Extraneuronal cholinergic system in lymphocytes. Pharmacol. Ther. 86, 29–48.
- Kawashima, K., Fujii, T., 2003a. The lymphocytic cholinergic system and its biological function. Life Sci. 72, 2101–2109.
- Kawashima, K., Fujii, T., 2003b. The lymphocytic cholinergic system and its contribution to the regulation of immune activity. Life Sci. 74, 675–696.
- Kawashima, K., Fujii, T., 2004. Expression of non-neuronal acetylcholine in lymphocytes and its contribution to the regulation of immune function. Front. Biosci. 9, 2063–2085.
- Kawashima, K., Yoshikawa, K., Fujii, Y.X., Moriwaki, Y., Misawa, H., 2007. Expression and function of genes encoding cholinergic components in murine immune cells. Life Sci. 80, 2314–2319.
- Martin, R.M., Brady, J.L., Lew, A.L., 1998. The need for IgG2c specific antiserum when isotyping antibodies from C57BL/6 and NOD mice. J. Immunol. Methods 212, 187–192.
- Nakamura, T., Matsui, M., Uchida, K., Futatsugi, A., Kusakawa, S., Matsumoto, N., Nakamura, K., Manabe, T., Taketo, M.M., Mikoshiba, K., 2004. M<sub>3</sub> muscarinic acetylcholine receptor plays critical role in parasymphathetic control of salivation in mice. J. Physiol. (London) 558, 561–575.
- Ohno-Shosaku, T., Matsui, M., Fukudome, Y., Shosaku, J., Tsubokawa, H., Taketo, M.M., Manabe, T., Kano, M., 2003. Postsynaptic M<sub>1</sub> and M<sub>3</sub> receptors are responsible for the muscarinic enhancement of retrograde endocannabinoid signaling in the hippocampus. Eur. J. Neurosci. 18, 109–116.
- Skok, M.V., Grailhe, R., Changeux, J.-P., 2005. Nicotinic receptors regulate B lymphocyte activation and immune response. Eur. J. Pharmacol. 517, 246–251.
- Szelenyi, J., Baratha, E., Hollan, S., 1982. Acetylcholinesterase activity of lymphocytes: an enzyme characteristic of T-cells. Br. J. Haematol. 50, 241–245.
- Tayebati, S.K., El-Assouad, D., Ricci, A., Amenta, F., 2002. Immunochemical and immunocytochemical characterization of cholinergic markers in human peripheral blood lymphocytes. J. Neuroimmunol. 132, 147–155.
- Van Ommen, R., Vredendaal, A.E., Savelkoul, H.F., 1994. Suppression of polyclonal and antigen-specific murine IgG<sub>1</sub> but not IgE response by neutralizing interleukin-6 *in vivo*. Eur. J. Immunol. 24, 1396–2403.
- Zimring, J.C., Kapp, L.M., Yamada, M., Wess, J., Kapp, J.A., 2005. Regulation of CD8<sup>+</sup> cytolytic T lymphocyte differentiation by a cholinergic pathway. J. Neuroimmunol. 164, 66–75.

# Enhanced serum antigen-specific IgG<sub>1</sub> and proinflammatory cytokine production in nicotinic acetylcholine receptor $\alpha 7$ subunit gene knockout mice

Yoshihito X. Fujii<sup>a</sup>, Hirofumi Fujigaya<sup>a</sup>, Yasuhiro Moriwaki<sup>a</sup>, Hidemi Misawa<sup>a</sup>,  
Tadashi Kasahara<sup>b</sup>, Sergei A. Grando<sup>c</sup>, Koichiro Kawashima<sup>a</sup>,

<sup>a</sup> Department of Pharmacology, Kyoritsu College of Pharmacy, Minato-ku, Tokyo 105-8512, Japan

<sup>b</sup> Department of Biochemistry, Kyoritsu College of Pharmacy, Minato-ku, Tokyo 105-8512, Japan

<sup>c</sup> Department of Dermatology, University of California, Irvine, CA, USA

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## Abstract

Human and murine immune cells such as mononuclear leukocytes consisting of mainly T and B cells, bone marrow derived dendritic cells (DCs) and macrophages all express various nicotinic acetylcholine (ACh) receptor (nAChR) subunits. Activated T cells and DCs have the ability to synthesize ACh by choline acetyltransferase, suggesting the role of non-neuronal cholinergic system expressed in immune cells in the regulation of immune cell function. Stimulation of human leukemic T and B cell lines with nicotine causes a transient Ca<sup>2+</sup>-signaling that is antagonized by  $\alpha$ -bungarotoxin, suggesting the involvement of  $\alpha 7$  subunit. Furthermore,  $\alpha 7$  nAChRs have been shown to negatively regulate synthesis and release of tumor necrosis factor (TNF)- $\alpha$  in macrophages. These findings suggest that immune cell function is regulated by its own non-neuronal cholinergic system, at least in part, via  $\alpha 7$  nAChR-mediated pathways. In the present study, we tested the role of  $\alpha 7$  nAChRs in the regulation of immune function by measuring total serum and antigen-specific IgG<sub>1</sub> and IgM, and production of TNF- $\alpha$ , gamma interferon (IFN- $\gamma$ ) and interleukin (IL)-6 in activated spleen cells of nAChR  $\alpha 7$  subunit gene knockout ( $\alpha 7$  KO) and wild-type C57BL/6J mice immunized with ovalbumin (OVA). We found that serum levels of total and anti-OVA-specific IgG<sub>1</sub> were significantly elevated in  $\alpha 7$  KO mice, though there were no significant differences in serum levels of total and anti-OVA-specific IgM between the two genotypes. Production of TNF- $\alpha$ , IFN- $\gamma$  and IL-6 in spleen cells was significantly facilitated in  $\alpha 7$  KO mice. Expression of AChE mRNA was not different between the two genotypes. These results suggest that  $\alpha 7$  nAChRs are involved in the regulation of cytokine production, through which modulates TNF- $\alpha$ , IFN- $\gamma$  and IL-6 productions, leading to modification of antibody production, but are not involved in expression of cholinergic components in immune cells.

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**Keywords:** Acetylcholine;  $\alpha 7$  subunit; IgG<sub>1</sub>; IFN- $\gamma$ ; IL-6; TNF- $\alpha$

## 1. Introduction

Human mononuclear leukocytes (MNLs) and leukemic T and B cell lines, and murine immune cells such as T and B cells, bone marrow derived dendritic cells (DCs) and macrophages express various nicotinic acetylcholine (ACh) receptor (nAChR) subunits as well as five distinct subtypes of muscarinic ACh receptors (M<sub>1</sub>–M<sub>5</sub> mAChRs) (see reviews by

Kawashima and Fujii, 2000, 2003a,b, 2004; Fujii and Kawashima, 2001; Kawashima et al., 2007). Furthermore, the findings that immunologically activated T cells and DCs have the ability to synthesize ACh by choline acetyltransferase (ChAT) suggest the role of non-neuronal cholinergic system expressed in immune cells in the regulation of immune function. In fact, we have shown recently that levels of total serum IgG<sub>1</sub> and antigen-specific IgG<sub>1</sub>, and interleukin (IL)-6 production are all diminished in M<sub>1</sub>/M<sub>5</sub> mAChR gene knockout (M<sub>1</sub>/M<sub>5</sub> KO) mice 1 wk after immunization with ovalbumin (OVA), though there is no change in serum levels of total serum IgM and antigen-specific IgM. These results suggested the involvement of M<sub>1</sub> and/or M<sub>5</sub> mAChRs in the regulation of immune

Corresponding author. 7-18-4 Nagatsuda Minamidai, Midori-ku, Yokohama 226-0018, Japan. Tel./fax: +81 45 983 5964.

E-mail addresses: koichiro-jk@piano.ocn.ne.jp, kawashima-ki@kyoritsu-ph.ac.jp (K. Kawashima).

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function, at least in part through altered production of the Th2 type cytokine IL-6, leading to modulation of antibody class switching from IgM to IgG<sub>1</sub> (Fujii et al., in press).

Stimulation of human leukemic T and B cell lines expressing nAChR 2, 5, 6, 7, 9, 10 and 2 and 4 subunits with nicotine or epibatidine elicited a transient Ca<sup>2+</sup>-signaling that was antagonized effectively by  $\alpha$ -bungarotoxin, suggesting that the 7 nAChRs are at least partly responsible for nicotine-induced Ca<sup>2+</sup>-signaling in lymphocytes (Kimura et al., 2003; Kawashima and Fujii, 2004). While the expression level and pattern of nAChR subunits may vary depending upon the cells' stage of development and the stimulus given during development, nAChR 5 and 7 subunits appear to be always expressed in T cells from C57BL/6J mice (Kuo et al., 2002). Collectively, these findings suggest the role of 7 subunit in the regulation of immune cell function. Nicotine as well as exposure to cigarette smoke induces suppression of various immunological parameters such as T cell-dependent antibody and T cell mitogenic responses (see a review by Sopori, 2002). Skok et al. (2005) found reduced pre-immune level of serum IgG with little change in IgM in 4, 7 or 2 KO mice and enhanced serum IgG response to primary immunization with cytochrome c in either 4 KO or 2 KO mice, suggesting that the role of nAChRs in the regulation of antibody synthesis in B cells. However, the role of 7 nAChRs in the regulation of humoral response to antigen has not been explored yet.

In the present study, in order to investigate the role played by non-neuronal cholinergic system in the regulation of in vivo immune function, focusing on 7 nAChRs, we examined the production of total serum and antigen-specific IgM and IgG<sub>1</sub> in nAChR 7 subunit gene knockout (7 KO) mice at 2 wk after immunization with ovalbumin (OVA). Furthermore, in order to investigate the role of 7 nAChRs in antigen-specific cytokine production, we determined levels of TNF- $\alpha$ , gamma interferon (IFN- $\gamma$ ) and IL-6 in OVA-activated spleen cell cultures.

## 2. Materials and methods

### 2.1. Animals

7 KO (*Acra7*-deficient, 7<sup>-/-</sup>) C57BL/6J background mice were bred from the founders' strain generated by Orr-Urterger et al. (1997). Four male and 3 female 10 to 15-wk-old 7 KO mice and 3 male and 4 female age-matched wild-type C57BL/6J were used for study.

### 2.2. Immunization and bleeding

Mice were immunized by intraperitoneal injection of 100  $\mu$ g of OVA (Sigma, St. Louis, MO, USA) mixed with  $5 \times 10^8$  CFUs of *Bordetella pertussis* (LSL, Tokyo, Japan) and Alu-Gel-S (Serva, Heidelberg, Germany). Two wk after the immunization, blood samples were obtained by cardiac puncture under deep anesthesia with ether. Serum was separated by centrifugation and stored in a deep freezer until assayed.

### 2.3. Determination of serum IgG<sub>1</sub> and IgM

#### 2.3.1. Total IgG<sub>1</sub> and anti-OVA-specific IgG<sub>1</sub>

Total serum IgG<sub>1</sub> levels were quantified by ELISA using 96-well microtiter plates coated with goat anti-mouse IgG<sub>1</sub> (Cat # A90-105A, Bethyl, Montgomery, TX, USA) and goat anti-mouse IgG<sub>1</sub> conjugated with horseradish peroxidase (Cat # A90-105P, Bethyl, Montgomery, TX, USA). Mouse monoclonal anti-OVA-specific IgG<sub>1</sub> (HYB 099-01, Antibodyshop, Gentofte, Denmark) was used as the standard.

Serum levels of anti-OVA-specific IgG<sub>1</sub> were determined by ELISA using 96-well microtiter plates coated with OVA and goat anti-mouse IgG<sub>1</sub> conjugated with horseradish peroxidase (Cat # A90-105P, Bethyl, Montgomery, TX, USA). Mouse monoclonal anti-OVA-specific IgG<sub>1</sub> (HYB 099-01, Antibodyshop, Gentofte, Denmark) served as the standard.

#### 2.3.2. Total IgM and anti-OVA-specific IgM

Serum concentrations of total IgM and anti-OVA-specific IgM were determined using a commercially available Mouse IgM ELISA Quantitation Kit (Bethyl, Montgomery, TX, USA) and a Mouse Anti-Ovalbumin IgM ELISA Kit (Cat. # 600-170-OGM, Alpha Diagnostic, San Antonio, TX, USA), respectively, according to manufacturer's instructions.

### 2.4. Secretion of TNF- $\alpha$ , IFN- $\gamma$ and IL-6 and expression of ChAT and AChE mRNAs in spleen cells

#### 2.4.1. Preparation of spleen cells

Mice were sacrificed by deep anesthesia with ether, after which the spleen was dissected out. Suspensions of single spleen cells were prepared by passing the cells through a nylon mesh, after which contaminating erythrocytes were removed by treatment with ammonium chloride lysis buffer. Cell surface expression of antigens was identified by monoclonal antibody staining, followed by flow cytometry using a FACSCalibur (Becton-Dickinson, Palo Alto, CA, USA) with analysis using CellQuest software (Becton-Dickinson). Spleen cells thus prepared consisted of 90% lymphocytes (30% CD4<sup>+</sup> and 15% CD8<sup>+</sup> T cells; 45% CD45<sup>+</sup> B cells) and 10% monocytes (CD14<sup>+</sup> cells).

#### 2.4.2. Determination of TNF- $\alpha$ , IFN- $\gamma$ and IL-6 secretion in spleen cells

Spleen cell samples were suspended in a 10% fetus bovine serum/RPMI 1640 culture medium ( $2 \times 10^6$  cells/mL) and then incubated with 100  $\mu$ g/mL OVA at 37 °C in a CO<sub>2</sub> incubator. The conditioned media were collected after 24 h or 48 h of culture to assess secretion of IL-6 or TNF- $\alpha$  and IFN- $\gamma$ , respectively. The levels of these cytokines were determined using commercially available ELISA kits (IFN- $\gamma$ , BD Sciences, San Diego, CA, USA; TNF- $\alpha$  and IL-6, R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

#### 2.4.3. Expression of AChE and ChAT mRNAs in spleen cells

2.4.3.1. *Extraction of total RNA from spleen cells.* After incubating spleen cells for 48 h in the presence of 100  $\mu$ g/mL

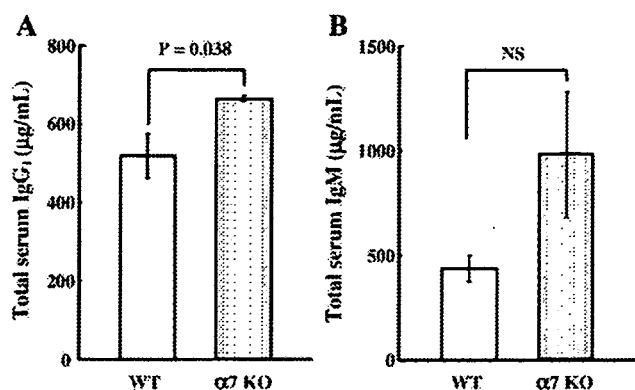


Fig. 1. Serum concentrations of total IgG<sub>1</sub> (A) and total IgM (B) two wk after immunizing  $\alpha 7$  KO and wild-type (WT) C57BL/6J mice with OVA. The bars represent means  $\pm$  SEM for seven mice.

OVA, total RNA was extracted from the cells using RNA Bee™ (TelTest, Friendswood, TX, USA) as described previously (Fujii et al., 1998). RNA concentration and quality were assessed based on 260 nm/280 nm absorbance ratios.

**2.4.3.2. First strand cDNA synthesis.** First-strand cDNA was prepared using 1  $\mu$ g of total RNA, 50 pmol of random primer p(dN)<sub>6</sub> and SuperScript™ II (Invitrogen, Carlsbad, CA, USA) in a 20- $\mu$ L reaction volume, as described elsewhere (Fujii et al., 1998).

**2.4.3.3. Conventional polymerase chain reaction (PCR) analysis.** Using a DNA Thermal Cycler (Perkin-Elmer Cetus, CT, USA), 2  $\mu$ L of cDNA was amplified in a 50- $\mu$ L reaction volume containing 25 pmol of the respective forward and reverse primers and AmpliTaq DNA polymerase (Takara, Shiga, Japan). The following sense and antisense oligonucleotide primers were custom synthesized at Sigma Genosys (Hokkaido, Japan) and used for PCR analysis of ChAT and AChE: 5'-GCAGTACTACAGACTCTTCTCATC-3' (ChAT sense), 5'-TCCAAGACAAAGAACTGGTTGC-3' (ChAT antisense), 5'-GCAGCAATATGTGAGCCTGA-3' (AChE sense), and 5'-GGTCGAACTGGTTCTTCCAG-3' (AChE anti-sense).

For ChAT and AChE, the amplification protocol entailed 40 cycles of denaturation at 95 °C for 30 s, annealing at 53–57 °C for 30 s and extension at 72 °C for 30 s. The PCR products present in 10  $\mu$ L of the reaction mixture were then separated according to size on 2% agarose gels and visualized by ethidium bromide staining. The sizes of the PCR products were estimated from the migration of DNA markers run concurrently.

All other reagents used were of reagent grade and purchased from commercial sources (e.g., Wako Pure Chemicals, Osaka, Japan).

**2.4.3.4. Real time PCR analysis.** After our conventional reverse transcription (RT)-PCR analysis confirmed the expression of AChE mRNA (see Results), we used real time PCR to determine the relative levels of AChE mRNA expression in  $\alpha 7$  KO and wild-type mice. The following sense and antisense oligonucleotide primers for the mouse gene encoding AChE

were designed with assistance of Sigma Genosys (Hokkaido, Japan): 5'-CGGAGGCTCTCATCAATACTGG-3' (AChE sense) and 5'-GGGACCCCGTAAACCAGAAAG-3' (AChE antisense).

The PCR was carried out using an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) and the TaqMan Universal Master Mix reagent (Applied Biosystems, Foster City, CA, USA) in accordance with the manufacturer's protocol. Briefly, 1  $\mu$ L of the diluted cDNA sample produced from 1  $\mu$ g of total RNA was added to 25  $\mu$ L of the PCR master mix. To correct for minor variations in mRNA extraction and reverse transcription, the gene expression values were normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), after which the AChE/GAPDH ratios obtained from  $\alpha 7$  KO mice were expressed as percentages of the mean value obtained from wild-type mice.

### 2.5. Statistical analysis

All values are expressed as means  $\pm$  SEM. Statistical comparisons between the  $\alpha 7$  KO and wild-type mice were carried out using an unpaired *t*-test. Values of  $P < 0.05$  were considered significant. In addition, linear regression analyses were performed to investigate whether there were any correlations among the respective immunoglobulin levels, cytokine secretion and/or expression of AChE mRNA.

## 3. Results

### 3.1. Serum IgG<sub>1</sub> and IgM

IgG<sub>1</sub> and IgM responses in  $\alpha 7$  KO mice were investigated by measuring both total serum and antigen-specific IgG<sub>1</sub> and IgM concentrations at 2 wk of immunization with OVA.

#### 3.1.1. Total serum IgG<sub>1</sub> and IgM

Total serum IgG<sub>1</sub> was significantly higher in  $\alpha 7$  KO than in wild-type mice ( $P = 0.038$ ) (Fig. 1A). Although total serum IgM in  $\alpha 7$  KO mice tended to be higher than that in wild-type

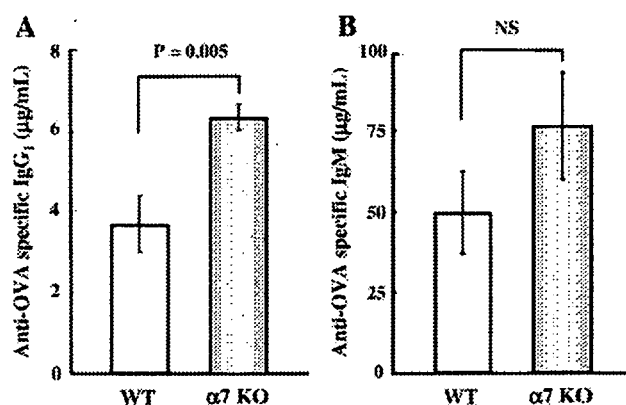


Fig. 2. Serum concentrations of anti-OVA-specific IgG<sub>1</sub> (A) and anti-OVA-specific IgM (B) two wk after immunizing  $\alpha 7$  KO and wild-type (WT) C57BL/6J mice with OVA. The bars represent means  $\pm$  SEM for seven mice.

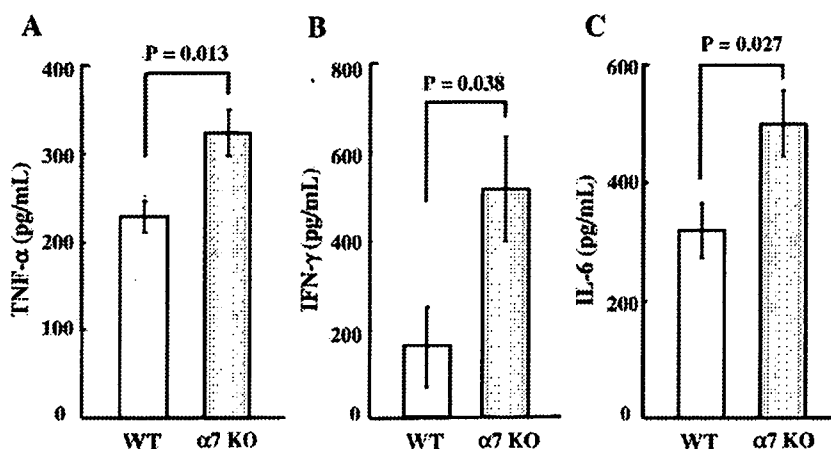


Fig. 3. Secretion of TNF- $\alpha$  (A), IFN- $\gamma$  (B) and IL-6 (C) in spleen cells from  $\alpha 7$  KO and wild-type (WT) C57BL/6J mice. Spleen cells were cultured with 100  $\mu$ g/mL OVA for 48 (A, B) or 24 (C) h. The bars represent means  $\pm$  SEM for seven mice.

mice, the difference was not statistically significant due to a large inter-individual variation (Fig. 1B).

### 3.1.2. Anti-OVA-specific serum IgG<sub>1</sub> and IgM

$\alpha 7$  KO mice had a significantly higher serum level of anti-OVA-specific IgG<sub>1</sub> than wild-type mice ( $P=0.005$ ) (Fig. 2A). The serum level of anti-OVA-specific IgM showed a tendency to increase in  $\alpha 7$  KO compared with the wild-type mice, but no significant difference was observed between the two genotypes because of a large inter-individual variation (Fig. 2B).

These findings suggest that  $\alpha 7$  nAChRs are involved in negative regulation of synthesis of IgG<sub>1</sub> in B cells.

### 3.2. TNF- $\alpha$ , IFN- $\gamma$ and IL-6 secretion from activated spleen cells

The possibility that the modified IgG<sub>1</sub> and IgM responses were related to cytokine production in  $\alpha 7$  KO mice was assessed by measuring TNF- $\alpha$ , IFN- $\gamma$  and IL-6 production in

antigen-stimulated spleen cells from immunized mice after 24 or 48 h of culture. OVA-activated spleen cells from  $\alpha 7$  KO mice secreted significantly more TNF- $\alpha$ , IFN- $\gamma$  and IL-6 than those from wild-type mice ( $P=0.013$ ;  $P=0.038$ ;  $P=0.027$ , respectively) (Fig. 3), suggesting the role of these cytokines in the enhancement of IgG<sub>1</sub> responses in  $\alpha 7$  KO mice.

### 3.3. Expression of ChAT and AChE mRNAs in OVA-activated spleen cells

Firstly, we detected expression of AChE mRNA in spleen cells from both  $\alpha 7$  KO and wild-type mice using conventional RT-PCR. However, no expression of ChAT mRNA was detected in either genotype (data not shown). Secondly, using real time PCR, we determined the level of AChE mRNA expression in  $\alpha 7$  KO, but did not find any difference between the two genotypes (Fig. 4).

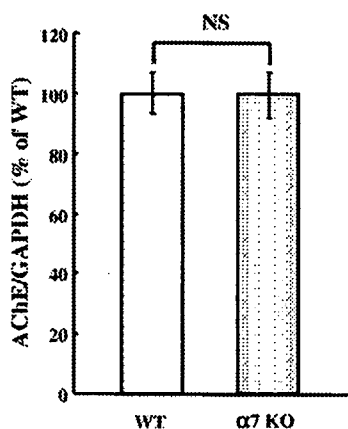


Fig. 4. Real time PCR analysis of AChE mRNA expression in spleen cells from  $\alpha 7$  KO and wild-type (WT) C57BL/6J mice. Spleen cells were cultured with 100  $\mu$ g/mL OVA for 48 h. The bars represent means  $\pm$  SEM ( $n=7$ ) for the levels of AChE mRNA normalized to the levels GAPDH mRNA and expressed a percentage of the mean levels in wild-type cells.

Table 1  
Results of linear correlation of analyses between A and B

A	B	P value	r
Anti-OVA specific IgG1	Total IgG1	0.026	0.591
Anti-OVA specific IgG1	TNF- $\alpha$	0.025	0.593
Anti-OVA specific IgG1	IFN- $\gamma$	0.035	0.565
Anti-OVA specific IgG1	IL-6	0.010	0.657
Anti-OVA specific IgM	Total IgM	0.002	0.745
Anti-OVA specific IgM	TNF- $\alpha$	0.760	–
Anti-OVA specific IgM	IFN- $\gamma$	0.943	–
Anti-OVA specific IgM	IL-6	0.361	–
Total IgG1	Total IgM	0.399	–
Total IgG1	TNF- $\alpha$	0.185	–
Total IgG1	IFN- $\gamma$	0.555	–
Total IgG1	IL-6	0.043	0.547
Total IgM	TNF- $\alpha$	0.796	–
Total IgM	IFN- $\gamma$	0.865	–
Total IgM	IL-6	0.117	–
TNF- $\alpha$	IFN- $\gamma$	0.001	0.832
TNF- $\alpha$	IL-6	0.211	–
IFN- $\gamma$	IL-6	0.494	–

$N=14$ .  $r$ , correlation coefficient; OVA, ovalbumin; IFN- $\gamma$ , gamma interferon; IL-6, interleukin-6; TNF- $\alpha$ , tissue necrosis factor- $\alpha$ .

### 3.4. Correlations between the measurements

Linear regression analyses revealed that TNF- $\alpha$  production in OVA-stimulated spleen cells correlated significantly with IFN- $\gamma$  [ $r=0.832$  ( $P=0.001$ )], confirming that IFN- $\gamma$  production in immune cells is regulated, at least in part, by TNF- $\alpha$  (Table 1). Anti-OVA-specific serum IgG<sub>1</sub> was significantly correlated with IL-6 [ $r=0.657$  ( $P=0.010$ )], TNF- $\alpha$  [ $r=0.593$  ( $P=0.025$ )] and INF- $\gamma$  [ $r=0.565$  ( $P=0.035$ )]. Significant correlations were also observed between total IgG<sub>1</sub> and anti-OVA-specific IgG<sub>1</sub> [ $r=0.591$  ( $P=0.026$ )], and between total IgM and anti-OVA-specific IgM [ $r=0.745$  ( $P=0.002$ )].

## 4. Discussion

The results of the present study demonstrate that  $\alpha 7$  nAChRs expressed in immune cells are involved in negative regulation of IgG<sub>1</sub> antibody production. The significant correlations of serum anti-OVA-specific IgG<sub>1</sub> with TNF- $\alpha$ , INF- $\gamma$  and IL-6 secreted from activated spleen cells suggest that  $\alpha 7$  nAChRs exert their effects on IgG<sub>1</sub> production via modification of cytokine synthesis in immune cells, at least in part. While both total serum IgM and anti-OVA-specific IgM were found to be increased in  $\alpha 7$  KO mice, their levels did not correlate with any cytokines in the present study, suggesting that the serum levels of total serum IgM and anti-OVA-specific IgM at 2 wk after the primary immunization are no more regulated by these cytokines produced in spleen cells.

The level of total serum IgG<sub>1</sub> in both  $\alpha 7$  KO and wild-type mice was more than 100 times that of anti-OVA-specific IgG<sub>1</sub> at 2 wk after the primary immunization, suggesting that the level of total serum IgG<sub>1</sub> reflects the pre-immune state. Since the level of total serum IgG<sub>1</sub> was significantly higher in  $\alpha 7$  KO than wild-type mice, it appears that the level of total serum IgG<sub>1</sub> is already higher in  $\alpha 7$  KO than wild-type mice even at the pre-immune state. Contrary to our findings, Skok et al. (2005) found a lower level of IgG in pre-immunization serum of 4, 7 or 2 KO mice compared with wild-type mice. Furthermore, they (2006) reported the role of  $\alpha 7$  nAChRs in the development of B cells in the bone marrow and proliferation of T and B cells in the spleen, suggesting the involvement of  $\alpha 7$  nAChRs in positive regulation of B cell function. The reasons for the discrepancies on the role of  $\alpha 7$  nAChRs in the regulation of immune cell function between the studies by Skok et al. (2005, 2006) and the present study remain to be clarified.

Using  $\alpha 7$  KO mice, Wang et al. (2003) showed that nicotine prevents lipopolysaccharide-induced septic shock by inhibiting synthesis and release of tumor necrosis factor (TNF)- $\alpha$ , a pro-inflammatory cytokine, in macrophages through  $\alpha 7$  nAChR-mediated pathways and confirmed essential role of  $\alpha 7$  nAChRs in the regulation of inflammatory responses. The spleen cells used in the present study consisted of T and B cells and monocytes containing macrophages and DCs. The higher TNF- $\alpha$  release from the antigen-stimulated spleen cells in  $\alpha 7$  KO than wild-type mice is in line with the results observed by Wang et al. (2003). Perhaps, ACh produced either by the antigen-activated T cells or DCs suppresses TNF- $\alpha$  release through stimulation of

$\alpha 7$  nAChRs in spleen cells of wild-type mice. However, the level of ChAT mRNA expression in the spleen cells was below the detection limit of RT-PCR with ethidium bromide staining. Vasoactive intestinal polypeptide (VIP) selectively increases the affinity of the agonist for nAChRs through action on VIP receptors, VPAC1 and VPAC2 (Liu et al., 2000). Spleen cells used in the present study express mRNAs for VIP as well as VPAC1 and VPAC2 (Kawashima et al., 2007). These findings suggest that even a small amount of ACh should be able to activate  $\alpha 7$  nAChRs and suppress TNF- $\alpha$  release in the presence of VIP.

Secreted mammalian Ly-6/urokinase plasminogen-type activator receptor-related protein (SLURP)-1, a member of the Ly-6/uPAR protein superfamily, has been isolated from human blood and urine (Adermann et al., 1999) and found to potentiate the amplitude of the ACh-induced macroscopic currents in *Xenopus* oocytes expressing the human  $\alpha 7$  nAChRs acting as an allosteric agonist (Chimienti et al., 2003). Arredondo et al. (2005) have shown that SLURP-1 competes with nicotine for binding to  $\alpha 7$  nAChRs on human keratinocytes and directly induces various biochemical and functional effects such as increases of caspase-3 and -8 activities and enhancement of proapoptotic activity, suggesting that SLURP-1 has the possibility to serve as an endogenous agonist for human keratinocyte  $\alpha 7$  nAChRs. Furthermore, Moriwaki et al. (2007) demonstrated the expression of mRNA for SLURP-1 in various tissues and organs including the spleen and thymus as well as T and B cells, DCs and macrophages of C57BL/6J mice. Collectively, these findings suggest that even a small amount of ACh can effectively activate  $\alpha 7$  nAChRs in the presence of SLURP-1 acting as an allosteric agonist, or that SLURP-1 itself acting as an agonist stimulates  $\alpha 7$  nAChRs, leading to suppression of synthesis and release of TNF- $\alpha$  in spleen cells under the present experimental conditions.

Antigen-activated spleen cell cultures of  $\alpha 7$  KO mice contained higher levels of TNF- $\alpha$ , IFN- $\gamma$  and IL-6 than those of the wild-type mice (Fig. 3). Macrophages, DCs and monocytes can collectively synthesize and release these cytokines in response to specific antigenic stimulation and subsequently become activated in response to them. The major source of the TNF- $\alpha$  in the present study appears to be splenic CD14<sup>+</sup> cells (monocytes, macrophages and DCs) comprised of 10% of spleen cells. Furthermore, activated CD4<sup>+</sup> T and B cells, comprised of 30% and 45% of spleen cells, respectively, can also synthesize TNF- $\alpha$ . TNF- $\alpha$  with IL-2 stimulates synthesis and release of INF- $\gamma$  from activated T cells and DCs, which, in turn, activates phagocytosis by macrophages, and facilitates antigen recognition by T and B cells, leading to facilitation of antibody synthesis (Becher et al., 1999). Exposure of activated CD4<sup>+</sup> and CD8<sup>+</sup> T cells, macrophages and DCs to TNF- $\alpha$  promotes synthesis of IL-6 that induces the final maturation of B cells into antibody-producing cells (Akira et al., 1993; Hirano, 1998; Kopf et al., 1998). These findings suggest that proinflammatory cytokines, such as TNF- $\alpha$ , IFN- $\gamma$  and IL-6 individually have stimulatory effects on the humoral responses to various antigenic stimuli (Khan et al., 2002). Significant correlations of anti-OVA-specific IgG<sub>1</sub> with TNF- $\alpha$ , IFN- $\gamma$  and IL-6 (Table 1)

support the above findings. Therefore, elevated total serum IgG<sub>1</sub> and anti-OVA-specific IgG<sub>1</sub>, and somewhat increases in total serum IgM and anti-OVA-specific IgM in 7 KO mice observed in the present study could be ascribed to the disinhibition of TNF- release from immune cells due to 7 nAChR deficiency leading to facilitation of IFN- and IL-6 production.

In our previous study with M<sub>1</sub>/M<sub>5</sub> KO mice (Fujii et al., in press), we found a slight but significant suppression of AChE gene expression, suggesting that either M<sub>1</sub> and/or M<sub>5</sub> mAChR signaling modulates AChE transcription, though the precise mechanism remained unclear. Since we did not find any difference in the level of AChE gene expression between the genotypes in the present study, AChE expression appears to be independent of 7 nAChRs.

In conclusion, 7 nAChRs are involved in negative regulation of immune cell function by inhibiting synthesis and release of proinflammatory cytokines, such as TNF-, IFN- and IL-6 in spleen cells, leading to suppression of IgG<sub>1</sub> production. The results of the present study along with our previous findings on the role of M<sub>1</sub> and/or M<sub>5</sub> mAChRs involved in positive regulation of IL-6 production (Fujii et al., in press) support the notion that non-neuronal cholinergic system expressed in immune cells is involved in the regulation of immune function via both nAChRs- and mAChRs-mediated pathways.

## References

- Adermann, K., Wattler, F., Wattler, S., Heine, G., Meyer, M., Forssmann, W.-G., Nehls, M., 1999. Structural and phylogenetic characterization of human SLURP-1, the first secreted mammalian member of the Ly-6/uPAR protein superfamily. *Protein Sci.* 8, 810–819.
- Akira, S., Taga, T., Kishimoto, T., 1993. Interleukin-6 in biology and medicine. *Adv. Immunol.* 54, 1–78.
- Arredondo, J., Chernyavsky, A.I., Webber, R.J., Grando, S.A., 2005. Biological effects of SLURP-1 on human keratinocytes. *J. Invest. Dermatol.* 125, 1236–1241.
- Becher, B., Blain, M., Giacomini, P.S., Antel, J.P., 1999. Inhibition Th1 polarization by soluble TNF receptor is dependent on antigen-presenting cell-derived IL-12. *J. Immunol.* 162, 684–688.
- Chimienti, F., Hogg, R.C., Plantard, L., Lehmann, C., Brakch, N., Fischer, J., Huber, M., Bertrand, D., Hohl, D., 2003. Identification of SLURP-1 as an epidermal neuromodulator explains the clinical phenotype of Mal de Meleda. *Hum. Mol. Genet.* 12, 3017–3024.
- Fujii, T., Kawashima, K., 2001. The non-neuronal cholinergic system: an independent non-neuronal cholinergic system in lymphocytes. *Jpn. J. Pharmacol.* 85, 11–15.
- Fujii, T., Yamada, S., Watanabe, Y., Misawa, H., Tajima, S., Fujimoto, K., Kasahara, T., Kawashima, K., 1998. Induction of choline acetyltransferase mRNA in human mononuclear leukocytes stimulated by phytohemagglutinin, a T-cell activator. *J. Neuroimmunol.* 82, 101–107.
- Fujii, Y.X., Tashiro, A., Arimoto, K., Fujigaya, H., Yoshikawa, K., Moriwaki, Y., Misawa, H., Fujii, T., Matsui, M., Kasahara, T., Kawashima, K., in press. Diminished antigen-specific IgG<sub>1</sub> and interleukin-6 production and acetylcholinesterase expression in combined M<sub>1</sub> and M<sub>5</sub> muscarinic acetylcholine receptor knockout mice. *J. Neuroimmunol.*
- Hirano, T., 1998. Interleukin 6 and its receptor: ten year later. *Int. Rev. Immunol.* 16, 249–284.
- Kawashima, K., Fujii, T., 2000. Extraneuronal cholinergic system in lymphocytes. *Pharmacol. Ther.* 86, 29–48.
- Kawashima, K., Fujii, T., 2003a. The lymphocytic cholinergic system and its biological function. *Life Sci.* 72, 2101–2109.
- Kawashima, K., Fujii, T., 2003b. The lymphocytic cholinergic system and its contribution to the regulation of immune activity. *Life Sci.* 74, 675–696.
- Kawashima, K., Fujii, T., 2004. Expression of non-neuronal acetylcholine in lymphocytes and its contribution to the regulation of immune function. *Front. Biosci.* 9, 2063–2085.
- Kawashima, K., Yoshikawa, K., Fujii, Y.X., Moriwaki, Y., Misawa, H., 2007. Expression and function of genes encoding cholinergic components in murine immune cells. *Life Sci.* 80, 2314–2319.
- Khan, A.Q., Shen, Y., Wu, Z.-Q., Wynn, T.A., Snapper, C.M., 2002. Endogenous pro- and anti-inflammatory cytokines differentially regulates an in vivo humoral response to *Streptococcus pneumoniae*. *Infect. Immun.* 70, 749–761.
- Kimura, R., Ushiyama, N., Fujii, T., Kawashima, K., 2003. Nicotine-induced Ca<sup>2+</sup> signaling and down-regulation of nicotinic acetylcholine receptor subunit expression in the CEM human leukemic T-cell line. *Life Sci.* 72, 2155–2159.
- Kopf, M., Herren, S., Wiles, M.V., Pepys, M.B., Kosco-Vilbois, M.H., 1998. Interleukin 6 influences germinal center development and antibody production via a contribution of C3 complement component. *J. Exp. Med.* 188, 1895–1906.
- Kuo, Y., Lucero, L., Michaels, J., DeLuca, D., Lukas, R.J., 2002. Differential expression of nicotinic acetylcholine receptor subunits in fetal and neonatal mouse thymus. *J. Neuroimmunol.* 130, 140–154.
- Liu, D.M., Cuevas, J., Adams, D.J., 2000. VIP and PACAP potentiation of nicotinic ACh-evoked currents in rat parasympathetic neurons is mediated by G-protein activation. *Eur. J. Neurosci.* 12, 2243–2251.
- Moriwaki, Y., Yoshikawa, K., Fukuda, H., Fujii, Y.X., Misawa, H., Kawashima, K., 2007. Immune system expression of SLURP-1 and SLURP-2, two endogenous nicotinic acetylcholine receptor ligands. *Life Sci.* 80, 2365–2368.
- Orr-Urterger, A., Goldner, F.M., Saeki, M., Lorenzo, I., Goldberg, L., De Biasi, M., Dani, J.A., Patrick, J.W., Beaudet, A.L., 1997. Mice deficient in the 7 neuronal nicotinic acetylcholine receptor lack -bungarotoxin binding sites and hippocampal fast nicotinic currents. *J. Neurosci.* 17, 9165–9178.
- Skok, M.V., Grailhe, R., Changeux, J.-P., 2005. Nicotinic receptors regulate B lymphocyte activation and immune response. *Eur. J. Pharmacol.* 517, 246–251.
- Skok, M.V., Grailhe, R., Agenes, F., Changeux, J.-P., 2006. The role of nicotinic acetylcholine receptors in lymphocyte development. *J. Neuroimmunol.* 171, 86–98.
- Sopori, M., 2002. Effects of cigarette smoke on immune system. *Nat. Rev. Immunol.* 2, 372–377.
- Wang, H., Yu, M., Ochani, M., Amella, C.A., Tanovic, M., Susaria, S., Li, J.H., Wang, H., Yang, H., Ulloa, L., Al-Abed, Y., Czura, C.J., Tracey, K.J., 2003. Nicotinic acetylcholine receptor 7 subunit is an essential regulator of inflammation. *Nature* 421, 384–388.



# Renal defects associated with improper polarization of the CRB and DLG polarity complexes in MALS-3 knockout mice

Olav Olsen,<sup>1</sup> Lars Funke,<sup>1</sup> Jia-fu Long,<sup>4</sup> Masaki Fukata,<sup>1</sup> Toshinari Kazuta,<sup>1</sup> Jonathan C. Trinidad,<sup>3</sup> Kimberly A. Moore,<sup>2</sup> Hidemi Misawa,<sup>1</sup> Paul A. Welling,<sup>5</sup> Alma L. Burlingame,<sup>3</sup> Mingjie Zhang,<sup>4</sup> and David S. Bredt<sup>1,6</sup>

<sup>1</sup>Departments of Physiology, <sup>2</sup>Cellular and Molecular Pharmacology, and <sup>3</sup>Pharmaceutical Chemistry, University of California, San Francisco, San Francisco, CA 94143

<sup>4</sup>Department of Biochemistry, Molecular Neuroscience Center, Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong

<sup>5</sup>Department of Physiology, University of Maryland School of Medicine, Baltimore, MD 21201

<sup>6</sup>Department of Integrative Biology, Eli Lilly and Company, Indianapolis, IN 46285

**K**idney development and physiology require polarization of epithelia that line renal tubules. Genetic studies show that polarization of invertebrate epithelia requires the crumbs, partition-defective-3, and discs large complexes. These evolutionarily conserved protein complexes occur in mammalian kidney; however, their role in renal development remains poorly defined. Here, we find that mice lacking the small PDZ protein mammalian LIN-7c (MALS-3) have hypomorphic, cystic, and fibrotic kidneys. Proteomic analysis defines MALS-3 as the only known core component of both the crumbs and discs large

cell polarity complexes. MALS-3 mediates stable assembly of the crumbs tight junction complex and the discs large basolateral complex, and these complexes are disrupted in renal epithelia from MALS-3 knockout mice. Interestingly, MALS-3 controls apico-basal polarity preferentially in epithelia derived from metanephric mesenchyme, and defects in kidney architecture owe solely to MALS expression in these epithelia. These studies demonstrate that defects in epithelial cell polarization can cause cystic and fibrotic renal disease.

## INTRODUCTION

Polarity is a physical attribute of most eukaryotic cells that is indispensable for their function. Generation and maintenance of cell polarity requires the active segregation of molecular components and imparts distinct properties to subcellular domains. Polarization occurs along a major axis in epithelia, neurons, and asymmetrically dividing cells. In epithelia, polarization relative to the tight junction functionally separates the apical and basolateral domains; neuronal polarization allows for differential development of dendrites and axons. During asymmetric cell division, a polarity cue in the mother cell directs distribution of cell fate determinants in daughter cells. In all these cases, failure to establish cell polarity compromises tissue differentiation and function.

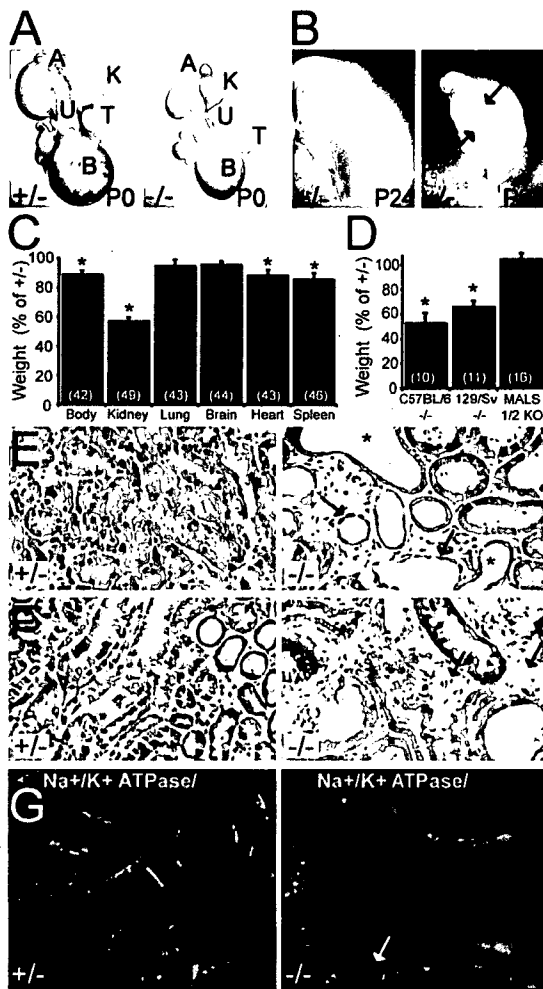
Sustained polarization of epithelial cells lining renal tubules is essential for kidney development and function (Campo et al., 2005). Tubules in the permanent kidney develop through reciprocal interactions between the ureteric bud and metanephric mesenchyme (Dressler, 2006). The ureteric bud invades the metanephros and undergoes a series of branching events that give rise to the collecting duct system. In response to signals released from ureteric bud tips, mesenchymal cells transform into polarized epithelia, which differentiate into the tubule cells along the remainder of the nephron. Epithelial cell polarization relies on tight junctions, which connect tubule cells, provide paracellular barriers to ion and fluid movement, and organize the membrane into apical and basolateral domains (Schneeberger and Lynch, 1992; Van Itallie and Anderson, 2004; Shin et al., 2006). Polarized expression of channels and transporters allows for vectorial transport of solutes along the nephron, resulting in physiological urine formation. Aberrations in epithelial cell polarity are implicated in the pathogenesis of renal cysts, renal fibrosis, and renal failure (Kalluri and Neilson, 2003; D.B. Lee et al., 2006); however, the molecular mechanisms underlying these complex diseases remain poorly understood.

O. Olsen and L. Funke contributed equally to this paper.

Correspondence to Olav Olsen: olav.olsen@ucsf.edu; or David S. Bredt: bredt@lilly.com

Abbreviations used in this paper: CRB, Crumbs; DLG, Discs large; LGL, lethal giant larvae; MAGUK, membrane-associated guanylate kinase; MALS-3, mammalian LIN-7c; MMKO, metanephric mesenchyme KO; PALS, proteins associated with LIN-7; PAR-3, partition-defective-3; PATJ, PALS-associated tight junction; SCRIB, scribble; UBKO, ureteric bud KO.

The online version of this article contains supplemental material.



**Figure 1. *MALS-3*<sup>−/−</sup> mice display renal defects.** (A) Dissected urinary tracts from P0 pups show significantly smaller kidneys in *MALS-3* knockout (□/□) mice as compared with heterozygote (□/□) littermates. A, adrenal gland; B, bladder; K, kidney; T, testis; U, ureter. (B) Kidneys from adult *MALS-3*<sup>−/−</sup> mice are hypoplastic, dysplastic, and cystic; arrows mark cysts. (C) Quantification of body and organ weight of adult *MALS-3*<sup>−/−</sup> mice and littermates. Kidneys from *MALS-3*<sup>−/−</sup> are significantly smaller (57.9% ± 1.8; *P* < 0.01). (D) *MALS-3*<sup>−/−</sup> mice backcrossed to 129/Sv or C57BL/6 for 8 generations show reduced kidney size (64.8% ± 3.2; *P* < 0.01 or 53.4% ± 8.5; *P* < 0.01, respectively). Mice lacking both *MALS-1* and *MALS-2* (*MALS* 1/2 KO) have normal kidneys. For C and D the number of animals is within the parentheses. (E) Hematoxylin and eosin stained kidney section from a 6-wk-old *MALS-3*<sup>−/−</sup> mouse shows normal arrangement of densely packed tubules (left). Kidney from a *MALS-3*<sup>−/−</sup> littermate shows marked tubulointerstitial changes, including dilatation of tubular lumina, tubular dedifferentiation, and fibrosis (right). Asterisks mark tubules with epithelia undergoing dedifferentiation, and arrows show dedifferentiated tubules. (F) Trichrome stained kidney section from a 6-wk-old *MALS-3*<sup>−/−</sup> mouse displays little or no collagen deposition (left). In contrast, a kidney from a *MALS-3*<sup>−/−</sup> littermate stains bright blue (arrows), revealing extensive fibrosis (right). (G) Immunolocalization of the Na<sup>+</sup>/K<sup>+</sup> ATPase shows basolateral membrane localization in renal tubules of *MALS-3*<sup>−/−</sup> mice (left). Polarized expression of the Na<sup>+</sup>/K<sup>+</sup> ATPase is lost (arrows) in renal tubules undergoing simplification in *MALS-3*<sup>−/−</sup> mice.

Genetic studies of invertebrates have identified two conserved tight junction PDZ protein complexes, the Crumbs (CRB) and partition-defective-3 (PAR-3) complexes, which contribute to cell polarity (Tepass et al., 1990; Etemad-Moghadam et al., 1995; Wattset al., 1996; Tabuse et al., 1998; Bachmann et al., 2001;

Hong et al., 2001; Djiane et al., 2005). CRB is a transmembrane protein (Tepass et al., 1990), whose C-terminus binds to the PDZ domain of PALS (Roh et al., 2002) or PATJ (Bhat et al., 1999). In turn, PALS and PATJ bind one another through heterodimerization of their L27 domains (Roh et al., 2002). The CRB complex is an apical membrane determinant that stabilizes the apical membrane cytoskeleton through interaction with β-spectrin and D-moesin (Medina et al., 2002). The second complex includes the PDZ protein PAR-3, which binds to atypical protein kinase C (aPKC) (Izumi et al., 1998), and the PDZ protein PAR-6 (Joberty et al., 2000; Lin et al., 2000). This PAR-3 complex excludes certain basolateral proteins from the apical domain via aPKC phosphorylation (Betschinger et al., 2003; Plant et al., 2003). The CRB and PAR-3 polarity complexes were originally thought to act independently, but physical and functional interactions between these complexes establish cell polarity (Hurd et al., 2003).

Genetic analyses of invertebrates also identified several basolateral proteins required for epithelial cell polarization. Discs large (*dlg*) (Woods and Bryant, 1991), lethal giant larvae (*lgl*) (Gateff and Schneiderman, 1969), and scribble (*scrib*) (Bilder and Perrimon, 2000; Bilder et al., 2000) were originally identified as tumor suppressor genes in *Drosophila*. Mutations in *dlg*, *lgl*, or *scrib* disrupt epithelial cell polarity and cause neoplastic overgrowth of tissues. DLG genetically interacts with the multi-PDZ protein SCRIB and the WD40 motif protein LGL (Bilder et al., 2000). Interplay between these basolateral polarity proteins and the apical polarity complexes establish and maintain cellular polarity. For example, LGL is phosphorylated by aPKC, thereby excluding LGL from the apical compartment (Betschinger et al., 2003; Plant et al., 2003).

The mammalian homologue of LIN-7 (MALS) can bind components from both the apical (Kamberov et al., 2000) and basolateral (Kaech et al., 1998; Lee et al., 2002) polarity complexes, suggesting an additional mode for interplay. Three MALS genes occur in mammals, and each is a small protein comprising an L27 and a PDZ domain (Butz et al., 1998; Borg et al., 1999; Jo et al., 1999). The L27 domain links MALS to either the CRB (Kamberov et al., 2000) or DLG (Lee et al., 2002) complex, but a role for MALS in these polarity complexes is unknown. Biochemical and cell biological experiments suggest a variety of possible roles for MALS in mammalian epithelial cell lines. MALS localizes to the basolateral membrane of Madin-Darby canine kidney (MDCK) cells (Perego et al., 1999; Olsen et al., 2002). By analogy to the role of LIN-7 in *Caenorhabditis elegans*, the PDZ domain of MALS may anchor receptors to the basolateral domain of MDCK cells (Straight et al., 2001; Shelly et al., 2003). Alternatively, MALS may stabilize PALS and mediate tight junction formation (Straight et al., 2006). Other studies suggest that MALS interacts with β-catenin and mediates organization of adherens junctions (Perego et al., 2000). Finally, MALS can regulate endocytosis or endosomal sorting in epithelial cell lines (Perego et al., 1999; Straight et al., 2001). Despite these numerous suggestions, genetic analyses have yet to identify functions for MALS in mammalian epithelia.

We characterize *MALS-3* knockout (□/□) mice and identify an essential role for *MALS-3* in defining polarity of renal

Table I. Renal function tests

Renal function	♂/♀	♂/♀
Uv (ml/day)	2.26 ± 1.3	5.13 ± 2.2*
Ccre (ul/min)	73 ± 16	60 ± 11*
UNaV (uEq/day)	106 ± 26	180 ± 23*
UKV(uEq/day)	374 ± 103	427 ± 59
Uvca (mg/day)	0.11 ± 0.04	0.15 ± 0.04
UPiV (mg/day)	1.22 ± 0.49	0.63 ± 0.08
FENa%	0.74 ± 0.15	1.2 ± 0.09*
FEK%	60 ± 14	78 ± 6

Creatine clearance (Ccr) was calculated from the plasma (Pcr), urine creatine (Ucr) concentrations, and the urine flow rate (V),  $Ccr = U \times V / P$ ; Fractional excretion is the amount excreted divided by the amount filtered.  $n = 4$  each. \*,  $P < 0.05$ ,  $t$  test.

epithelial cells. *MALS-3<sup>β/β</sup>* mice have hypomorphic kidneys characterized by numerous cysts and Abrosis. These developmental defects owe to a loss of polarity specifically in epithelia derived from the metanephric mesenchyme. Immunoprecipitation proteomics analysis reveals MALS-3 binds to the CRB tight junction and DLG basolateral complexes. Biochemical studies show the L27 domain of MALS-3 assembles and stabilizes these complexes. In proximal tubule cells from MALS-3 mutant mice, the CRB complex is lost from tight junctions and DLG mislocalizes to tight junctions. These studies demonstrate that MALS-3 organizes these two discrete polarity complexes and that disruption of epithelial cell polarity can result in renal agenesis, cysts, and Abrosis.

## RESULTS

### Kidney pathology in *MALS-3<sup>β/β</sup>* mice

Kidneys from neonatal and adult *MALS-3<sup>β/β</sup>* mice are noticeably smaller than those from heterozygous (♂/♀) littermates (Fig. 1, A and B), and ~15% of *MALS-3<sup>β/β</sup>* display unilateral renal agenesis (unpublished data). Furthermore, *MALS-3<sup>β/β</sup>* kidneys are studded with numerous cysts (Fig. 1 B; and Fig. S1 A, available at <http://www.jcb.org/cgi/content/full/jcb.200702054/DC1>). Most cysts (8 of 11) fail to express tubule segment-specific markers, only 1 of 11 cysts stained with a lectin that labels proximal tubules (Fig. S1 B). Kidneys from *MALS-3<sup>β/β</sup>* are dramatically reduced in weight relative to *MALS-3<sup>β/β</sup>* (Fig. 1 C). Whereas the kidney is reduced by nearly 45%, only a ~10% reduction in the size of heart, spleen, and overall body weight is observed. Renal hypoplasia occurs in *MALS-3<sup>β/β</sup>* mice backcrossed to either the 129/Sv or C57BL/6 strain and is specific to *MALS-3<sup>β/β</sup>*, as kidneys from mice lacking both MALS-1 and MALS-2 are normal in size (Fig. 1 D).

Microscopic analysis of adult and newborn *MALS-3<sup>β/β</sup>* kidneys reveals dramatic cellular abnormalities in kidney architecture (Fig. 1 E; Fig. S1 C). *MALS-3<sup>β/β</sup>* kidneys display marked tubulointerstitial changes including tubular dilatation and dedifferentiation/simplification (Fig. 1 E). Trichrome staining reveals interstitial Abrosis (Fig. 1 F; Fig. S1 D), a condition characterized by the accumulation of extracellular matrix proteins and Abroblasts, which is a hallmark of end-stage renal disease (Kalluri and Neilson, 2003; J.M. Lee et al., 2006). Renal cysts and Abrosis in *MALS-3<sup>β/β</sup>* are accompanied by a

Table II. Plasma chemistry

Plasma	♂/♀	♂/♀
Na (mEq/l)	151 ± 2.5	151 ± 3.4
K (mEq/l)	6.6 ± 0.58	6.33 ± 0.32
Cl (mEq/l)	113 ± 3.7	110 ± 3.09
Creatinine (mg/dl)	0.32 ± 0.04	0.3 ± 0.0
Ca (mg/dl)	8.86 ± 0.3	9.6 ± 0.12
Pi (mg/dl)	8.86 ± 0.3	7.9 ± 0.35
BUN (mg/dl)	27 ± 6.69	40 ± 5.24*

\*,  $P < 0.05$ ,  $t$  test.

loss of epithelial cell polarity. The  $Na^{\beta}/K^{\beta}$  ATPase, which is normally localized basolaterally, becomes diffusely localized in dedifferentiated tubular epithelia (Fig. 1 G).

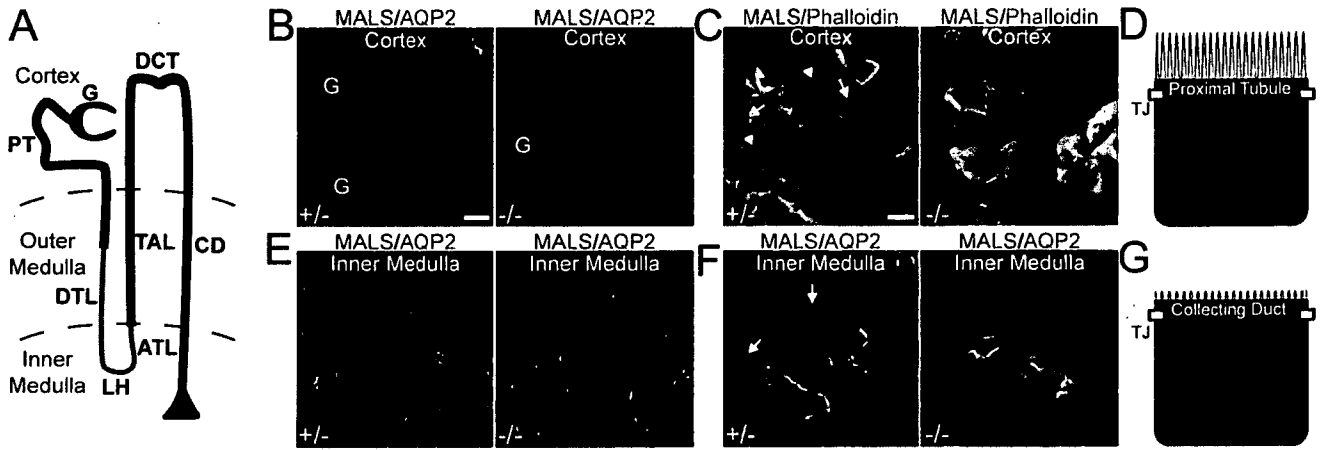
*MALS-3<sup>β/β</sup>* mice also manifest defects in renal function (Table I). Compared with *MALS-3<sup>β/β</sup>*, *MALS-3<sup>β/β</sup>* mice exhibit a considerable increase in urine output and sodium excretion. Creatine clearance, a measure of glomerular function, was modestly decreased whereas blood urea nitrogen (BUN) was elevated (Table II). This is likely a consequence of extracellular fluid volume contraction resulting from the polyuria. Urine concentrating ability, measured as the response to 12-h water deprivation and vasopressin injection after a high water load, was also impaired; urine osmolarity only increased 3 ± 0.9-fold (1,024 ± 490 mOsm/l change) in *MALS-3<sup>β/β</sup>* mice compared with 7.2 ± 0.9-fold (2,764 mOsm/l vs 332 mOsm/l) in *MALS-3<sup>β/β</sup>* (Fig. S1 E). Together with the histopathological observations, these results are reminiscent of nephronophthisis, the most common genetic disorder of progressive renal failure in children.

MALS-3 localizes to epithelial cells along the length of the nephron

To determine the pathogenesis of these kidney defects, the cellular distribution of MALS-3 protein was examined in kidney sections. MALS-3 is present in epithelial cells of renal tubules but is absent from glomeruli (Fig. 2). In the kidney cortex, MALS-3 occurs in collecting duct epithelia that label for aquaporin-2 (Fig. 2 B) and proximal tubule epithelia that stain intensely for phalloidin (Fig. 2 C). In proximal tubule cells, MALS-3 localizes to the basolateral membrane and the tight junction (Fig. 2, C and D; Fig. S2 A, available at <http://www.jcb.org/cgi/content/full/jcb.200702054/DC1>). In the inner medulla, MALS-3 is present in tubule cells of the loop of Henle and collecting duct (Fig. 2 E). In collecting duct epithelia, MALS-3 is restricted to the basolateral membrane and is not detected at the tight junction (Fig. 2, F and G). As expected, MALS-3 staining is absent in *MALS-3<sup>β/β</sup>* mice (Fig. 2, B, C, E, and F; and Fig. S2 A).

MALS-3 interacts with apical and basolateral polarity complexes

To define the proteins associated with MALS-3, we conducted immunoprecipitation proteomics. Immunoprecipitation of MALS-3 from adult *MALS-3<sup>β/β</sup>* kidney homogenates shows a series of specific protein bands that are absent from *MALS-3<sup>β/β</sup>* immunoprecipitates (Fig. 3 A). As determined by mass spectrometry, MALS-3 interacts with several proteins found in complexes

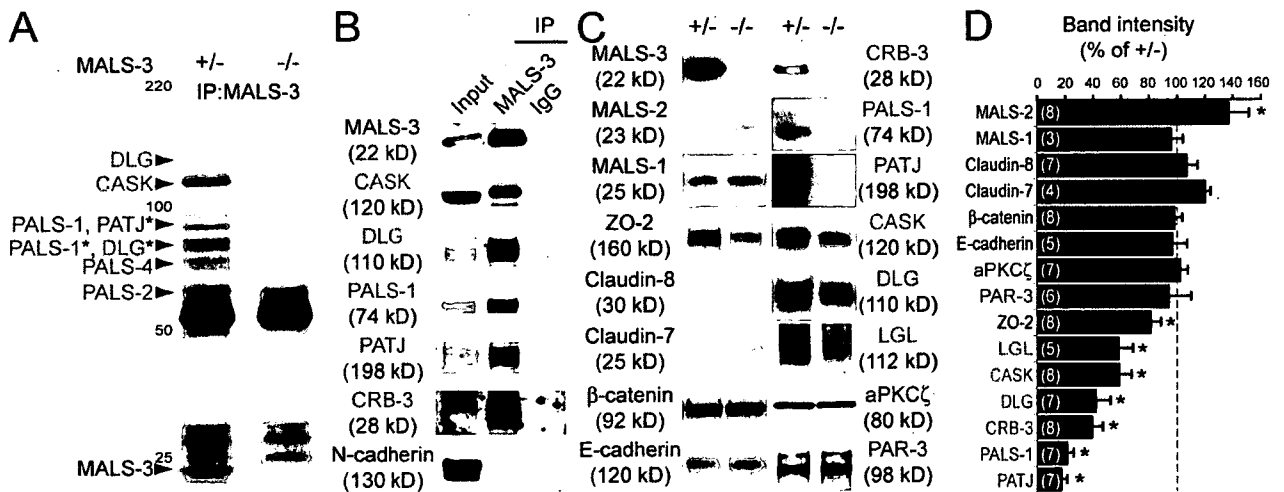


**Figure 2. MALS-3 localizes at the basolateral membrane and tight junction of renal epithelia.** (A) Schematic depicting the arrangement of tubular segments in the kidney. ATL, ascending thin limb; CD, collecting duct; DCT, distal convoluted tubule; DTL, descending thin limb; G, glomerulus; LH, loop of Henle; PT, proximal tubule; TAL, thick ascending limb. (B) Low magnification fluorescent images from the cortex of kidney sections from a 6-wk-old mouse reveal that MALS-3 (red) primarily localizes to the basolateral membrane of tubular structures. MALS-3 staining is absent from *MALS-3<sup>fl/fl</sup>*. AQP2 (green) specifically labels collecting ducts. (C) High magnification of proximal tubules shows a prominent brush border (phalloidin, green). DAPI (blue) stains nuclei. In proximal tubules, MALS-3 (red) is at both the basolateral membranes (arrowheads) and the tight junctions (arrows). (D) Cartoon summarizing MALS-3 localization in proximal tubules. Brush border and tight junction (TJ) are shown in green and yellow, respectively. (E and F) Low and high magnification images of the inner medulla show basolateral localization (arrows) of MALS-3 in collecting ducts (AQP2 positive tubules; green). (G) Cartoon depicting AQP2 (green) and MALS-3 (red) localizations in collecting ducts. Bars are 50  $\mu$ m in B and E; 10  $\mu$ m in C and F.

that determine cell polarity. MALS-3 immunoprecipitates contain DLG and CASK, proteins that localize to the basolateral surface, and PALS-1, PALS-2, PALS-4 (Mpp7), and PATJ, proteins that localize to tight junctions. Western blotting confirmed all of these interactions and demonstrated coimmunoprecipitation of CRB-3, the prototypical tight junction polarity protein, with similar recovery (Fig. 3 B).

To characterize the role of MALS-3 in these polarity complexes, we first assessed protein levels in *MALS-3<sup>fl/fl</sup>* kidneys. Strikingly, levels of all components of the CRB-3 tight junction complex are drastically diminished in *MALS-3<sup>fl/fl</sup>* kidneys (Fig. 3 C).

CRB-3 itself is decreased by 60% and PALS-1 and PATJ are each decreased by  $\approx$  80% (Fig. 3, C and D). In contrast, aPKC $\zeta$  and PAR-3, members of a distinct tight junction polarity complex, were unchanged in *MALS-3<sup>fl/fl</sup>*. Levels of basolateral proteins, LGL, CASK, and DLG are also significantly decreased (Fig. 3, C and D). Structural proteins of the tight junction, claudin-7 and -8, are unaffected in *MALS-3<sup>fl/fl</sup>*, while ZO-2 is modestly decreased. E-cadherin and  $\beta$ -catenin of the adherens junctions are also unchanged (Fig. 3, C and D). MALS-2 protein is up-regulated in kidneys of *MALS-3<sup>fl/fl</sup>*, which has also been noted in brains of MALS mutants (Misawa et al., 2001).



**Figure 3. MALS-3 interacts with both apical and basolateral polarity complexes in the kidney.** (A) Silver staining of kidney extracts immunoprecipitated with anti-MALS-3 antibody shows a series of proteins from *MALS-3<sup>fl/fl</sup>* homogenates that are absent from *MALS-3<sup>fl/fl</sup>* precipitates. (B) Western blotting of extracts immunoprecipitated with anti-MALS-3 antibody confirms specific association of CASK, DLG, PALS, PATJ, and CRB-3 with MALS-3. (C) Kidney homogenates from *MALS-3<sup>fl/fl</sup>* and *MALS-3<sup>fl/fl</sup>* mice immunoblotted for numerous proteins associated with MALS-3 and with epithelial cell junctions. (D) Quantification of protein levels in C. The number of blots quantified for each protein is in parentheses. In A–D, proteins of the CRB, PAR-3, and DLG complexes are shown in red, blue, and green, respectively. Asterisks denote P values < 0.05.

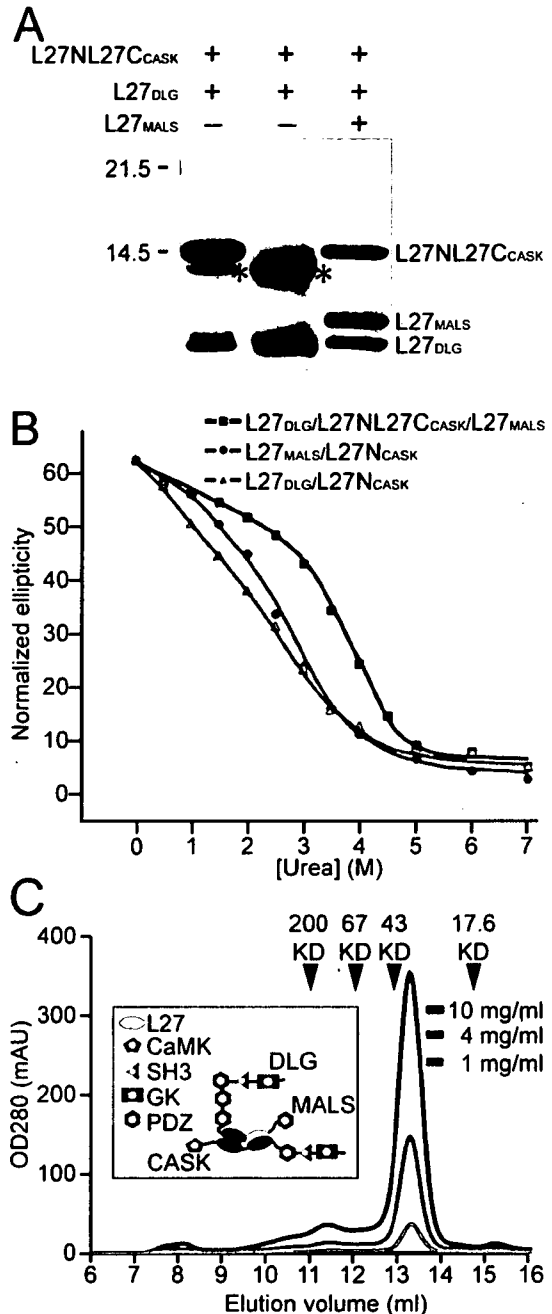
**MALS-3 L27 domain mediates assembly of CRB and DLG complexes**

Through L27 domain interactions, MALS-3 associates directly with CASK and PALS (Kaech et al., 1998; Kamberov et al., 2000; Lee et al., 2002), which in turn bind to DLG and PATJ (Lee et al., 2002; Roh et al., 2002), respectively. Interestingly, levels of DLG and PATJ, which do not directly bind to MALS-3, are reduced to a greater or similar extent as CASK and PALS (Fig. 3, C and D). This suggests that MALS-3 may mediate cooperative assembly of these L27 domain complexes. To explore this biochemically, the L27 domains of CASK and DLG were expressed in the absence of the MALS L27 domain in bacteria. Under these conditions, the CASK/DLG binary complex is substantially degraded upon isolation and further deteriorated within 24 h at 4°C (Fig. 4 A). Inclusion of the MALS L27 domain promotes formation of a MALS/CASK/DLG L27 complex that is stable for several days at 35°C (Fig. 4 A). Furthermore, the MALS/CASK/DLG ternary complex was more resistant to urea denaturation than either the MALS/CASK or DLG/CASK binary complexes (Fig. 4 B). MALS also stabilized the MALS/PALS/PATJ L27 complex (unpublished data). The oligomeric nature of protein complexes containing four L27 domains has not been determined. NMR showed that the isolated CASK and DLG L27 domains form a “dimer of dimers” structure (Feng et al., 2004), which might imply high order oligomerization of L27 domain complexes containing three proteins. However, gel filtration analysis shows that the MALS/CASK/DLG complex elutes as a single peak corresponding to ~38 kD (Fig. 4 C), implying a 1:1:1 stoichiometry. Similarly, the tandem PALS L27 domains elute with MALS L27 and PATJ L27 as a single peak corresponding to a 1:1:1 stoichiometry (unpublished data).

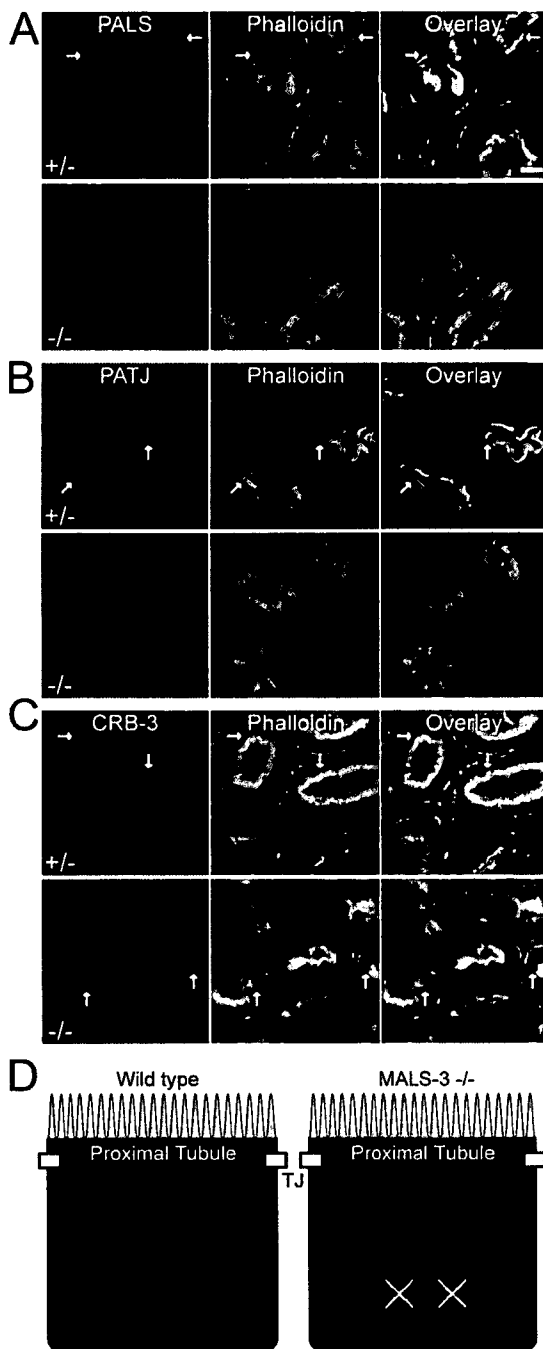
**The CRB-3/PATJ/PALS polarity complex requires MALS-3**

We next asked whether loss of MALS-3 affects the apico-basal polarization of its interaction partners. PALS, PATJ, and CRB-3 are enriched at tight junctions of proximal tubule epithelia (Fig. 5, A–D). Consistent with Western blotting results, PALS and PATJ staining is lost from tight junctions in *MALS-3<sup>β/β</sup>* (Fig. 5, A, B, and D); CRB-3 staining is reduced, and the remaining protein concentrates abnormally in punctate vesicles in the subapical region (Fig. 5, C and D). These major defects are not observed in collecting duct. In collecting duct epithelia, PALS and PATJ staining is reduced, but not lost, at the tight junction of the knockout, and CRB-3 localization appears normal (Fig. S3, A–D; available at <http://www.jcb.org/cgi/content/full/jcb.200702054/DC1>). Molecular compensation does not account for the localization of the CRB complex in *MALS-3<sup>β/β</sup>* collecting duct epithelia, as MALS-1 and -2 are not detected (Fig. S3 E).

Loss of MALS-3 specifically disrupts the CRB complex. In proximal tubule and collecting duct epithelia, aPKC, PAR-3, and ZO-1 remain at the tight junction (Fig. S3 H, Fig. S4, A, B, and D; and unpublished data). Other junction-associated proteins are also unaltered in renal epithelia from *MALS-3<sup>β/β</sup>* (Fig. S4, E and F).



**Figure 4. The L27 domain of MALS mediates cooperative formation of a stable MALS/CASK/DLG ternary complex.** (A) Purification of a recombinant binary complex containing the single L27 domain of DLG and the tandem L27 domains of CASK shows degradation (asterisk, lane 1) and further degrades over 24 h at 4°C (asterisk, lane 2). Co-expression of MALS L27 domain stabilizes the MALS/CASK/DLG complex (lane 3), which does not degrade after 3 d at 35°C. (B) Comparison of the urea-induced denaturation profile of the L27<sub>DLG</sub>/L27<sub>NL27</sub><sub>CASK</sub>/L27<sub>MALS</sub> complex with those of the (L27<sub>MALS</sub>/L27<sub>N<sub>CASK</sub>) and (L27<sub>N<sub>CASK</sub>/L27<sub>DLG</sub>) complexes. The ellipticities of each spectrum at 222 nm were used to construct the denaturation curves. The MALS/CASK/DLG ternary complex is more stable than the two binary domain complexes. (C) The tandem L27 domains of CASK assemble MALS and DLG into a stable 1:1:1 MALS/CASK/DLG ternary complex with a molecular mass of ~38 kD over a wide concentration range. Cartoon inset shows stoichiometry and molecular oligomerization of the MALS/CASK/DLG complex.</sub></sub>



**Figure 5. Disruption of the PALS/PATJ/CRB-3 polarity complex in renal epithelia lacking MALS-3.** In 6-wk-old *MALS-3<sup>+/±</sup>* mice, PALS (A, top), PATJ (B, top), and CRB-3 (C, top) localize to the tight junction (arrows) below the apical brush border (phalloidin, green) of proximal tubule epithelia. In *MALS-3<sup>-/-</sup>* mice, PALS (A, bottom) and PATJ (B, bottom) are no longer detected in proximal tubules. Most proximal tubules in *MALS-3<sup>-/-</sup>* mice also lack CRB-3 (C, bottom), but some show a sub-apical punctate pattern, reminiscent of apical endosomes (arrows). (D) Cartoon summarizing the localization of the PALS/PATJ/CRB-3 complex (red) in wild-type proximal tubules (left) and the disruption of the complex in proximal tubule epithelia lacking MALS-3 (right). Bar = 10  $\mu$ m.

**MALS-3 mediates apical repulsion of DLGs**  
 The distribution DLG undergoes complex changes in *MALS-3<sup>+/±</sup>* (Fig. 6). Normally, DLG is predominantly distributed basolaterally

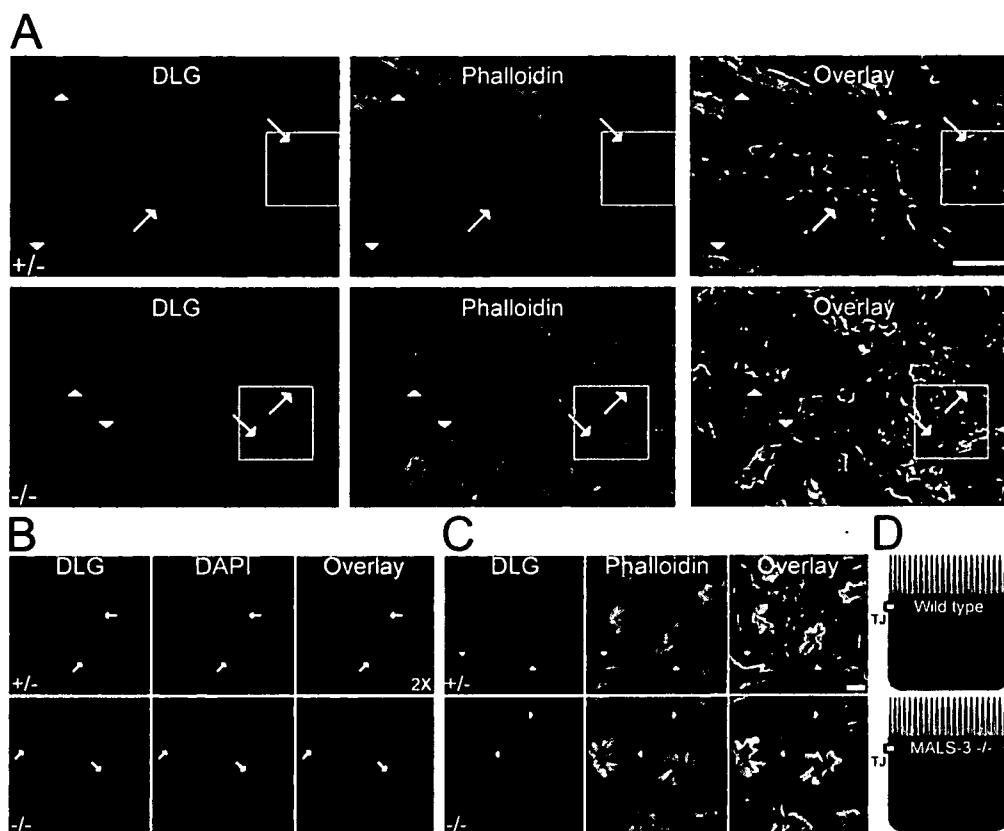
in proximal tubule epithelia (Fig. 6, A, C, and D) and in more distal tubular segments of the nephron (Fig. 6, A and B; Fig. S3 F). Weaker tight junction and cytoplasmic staining is also observed in control animals (Fig. 6 C). In *MALS-3<sup>+/±</sup>*, DLG is lost from the basolateral surface and becomes abnormally concentrated at tight junctions of epithelia in proximal tubules (Fig. 6, A, C, and D; and Fig. S2 B) and in more distal tubules (Fig. 6, A and B; Fig. S3 F). Other basolateral polarity proteins, scribble and LGL, remain concentrated along the basolateral membrane (Fig. S4 C, Fig. S3 G; and unpublished data).

#### Kidney organogenesis in *MALS-3<sup>-/-</sup>*

The resemblance of the *MALS-3* null phenotype to human nephronophthosis raises the possibility that MALS-3 may be involved in a common pathway with the six known nephronophthosis genes (NPHP1–6), which affect the organization and/or development of primary cilia (Torres et al., 2007). Consistent with this idea, CRB-3 localizes to cilia and *C. elegans* lacking *crb2b*, a CRB-3 homologue, exhibit renal hypoplasia, cysts, and tubular dilatation. Renal abnormalities in *crb2b* mutants are associated with malformation and dysfunction of primary cilia (Omori and Malicki, 2006). A conserved role for CRB-3 in cilia formation in mammalian epithelia has been demonstrated (Fan et al., 2004). Although CRB-3 is lost from the tight junction in renal epithelia lacking MALS-3, we found that CRB-3 localizes properly to primary cilia (Fig. 7 B), suggesting an alternative mechanism for targeting CRB-3 to cilia. Consistent with this hypothesis, PALS and PATJ are not detected in cilia (unpublished data) and the morphology of primary cilia appears unaltered in *MALS-3<sup>+/±</sup>* mice (Fig. 7 A). Analysis of EST databases (<http://www.ncbi.nlm.nih.gov>) revealed an alternatively spliced variant of CRB-3 (Fig. 7 C). The resulting protein product, CRB-3b, shares identical extracellular and transmembrane domains with canonical CRB-3, but has a divergent cytoplasmic tail. Importantly, the splicing variant results in a longer C-terminal tail that lacks the PDZ ligand (Fig. 7 C) required for PALS/PATJ interaction (Bhat et al., 1999; Roh et al., 2002). Interestingly, the CRB-3 antibody recognizes a doublet in kidney homogenates, with the larger product, likely CRB-3b, being more moderately reduced in *MALS-3<sup>+/±</sup>* (Fig. 3 C). Normalcy of ciliary structure and CRB localization do not exclude that more specific aspects of ciliary function are compromised in these animals (Hildebrandt and Zhou, 2007).

Dysfunction of primary cilia and associated centrosomes can impair cell proliferation (Simons and Walz, 2006) and thereby reduce kidney size in *MALS-3* mutants. To determine whether cell proliferation is altered in *MALS-3<sup>+/±</sup>* mice, mitotic cells in kidneys from E14.5 embryos were labeled using anti-phosphohistone3 antibody. We found no difference in cell proliferation between *MALS-3<sup>+/±</sup>* ( $0.388 \pm 0.013$ ) and *MALS-3<sup>-/-</sup>* ( $0.375 \pm 0.013$ ;  $P = 0.61$ ) littermates (Fig. 8, A and B), excluding reduced cell division as the cause for the smaller kidney size.

Next, we examined whether changes in apoptosis/cell death contribute to this phenotype. Apoptotic cells are found in nephrogenic regions (Koseki et al., 1992) of E14.5 kidneys from both control and *MALS-3<sup>+/±</sup>* littermates (Fig. 8 C). However, abnormal cell death was detected in cells underlying the renal capsule in *MALS-3<sup>+/±</sup>* kidneys (Fig. 8, C and D), resulting in a



**Figure 6. DLG mislocalizes to the tight junction in renal epithelia lacking MALS-3.** (A, top) At the medullary boundary of a  $MALS3^{+/+}$  kidney, DLG strongly labels the basolateral membranes of tubules (arrows) and faintly stains the tight junction of proximal tubule segments (arrowheads). Proximal tubule brush border is stained with phalloidin (green). (A, bottom) Basolateral localization of DLG is lost in renal epithelia lacking MALS-3 (arrows and arrowheads, as above) and replaced by staining at the tight junction. (B, top) Higher magnification of A shows basolateral localization of DLG (arrows). (B, bottom) Epithelia lacking MALS-3 show specific loss of DLG at the basolateral membrane and accumulation of DLG at the tight junction. (C, top) High magnification images of proximal tubules in the cortex show basolateral (arrowheads) and tight junction staining for DLG. (C, bottom) Proximal tubules of  $MALS3^{-/-}$  mice have a discrete loss of DLG from the basolateral membrane (arrowheads). (D) Cartoon summarizing the localization of DLG (red) in control renal epithelia and epithelia lacking MALS-3. Bar = 50  $\mu$ m in A and 10  $\mu$ m in C.

10-fold increase in apoptotic signal from  $MALS3^{+/+}$  (5.80  $\pm$  0.76) as compared with  $MALS3^{-/-}$  kidneys (0.42  $\pm$  0.19;  $P < 0.007$ ). These changes are detected as early as E12.5 (unpublished data), indicating that increased apoptosis causes reduced kidney size in  $MALS3^{-/-}$  mice.

**MALS-3 polarizes epithelia derived from the metanephric mesenchyme**

The profound disruption of the CRB-3 complex in proximal tubule cells and the normalcy of PALS and CRB-3 localization in the collecting duct suggest that MALS-3 serves distinct functions in these two epithelial cell types. Proximal tubule and collecting duct cells derive from different tissue types (Dressler, 2006). During embryonic development, the ureteric bud extends from the Wolffian duct, invades the metanephric mesenchyme, and induces mesenchymal to epithelial cell transition. Tubules derived from metanephric mesenchyme extend from the glomerulus to the distal tubule, whereas the collecting duct derives from the ureteric bud (Fig. 9 A).

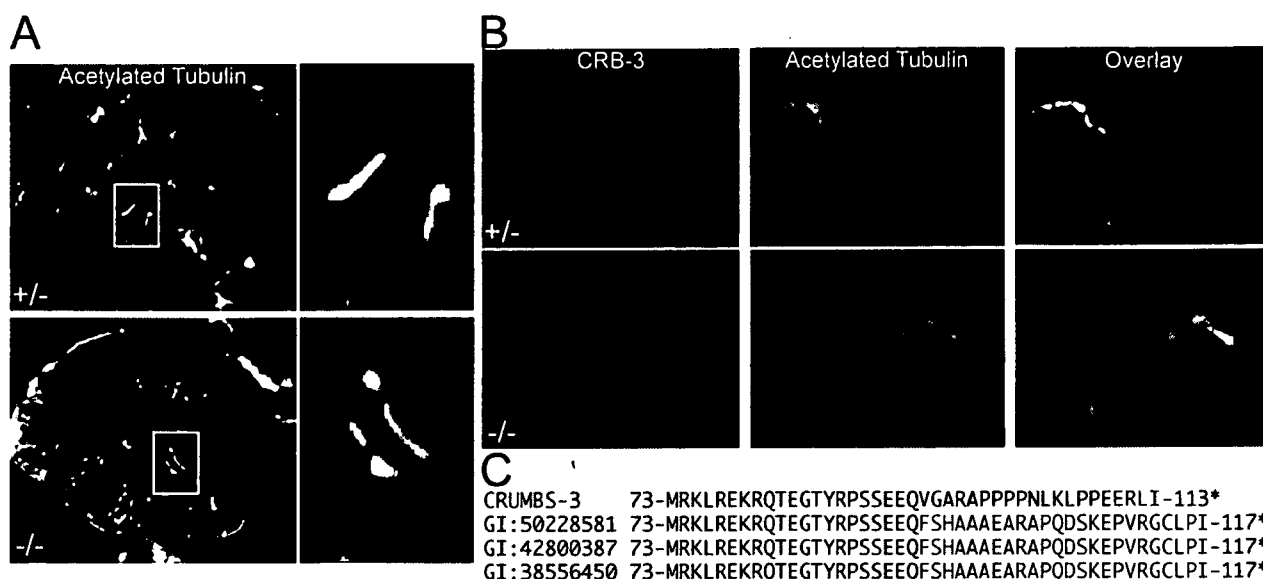
To assess the role of MALS-3 in each of these embryonic tissues, a new  $MALS3^{lox}$  allele was created (Fig. S5, A–C, available at <http://www.jcb.org/cgi/content/full/jcb.200702054/DC1>).

Breeding these  $MALS3^{lox/lox}$  mice to transgenics that express cre recombinase under the control of the HoxB7 or Pax3 promoter (Fig. 9 B) allows deletion of  $MALS3$  specifically from ureteric bud ( $MALS3^{lox/lox}; HoxB7-Pro-cre$ ) [UBKO] (Yu et al., 2002) or metanephric mesenchyme ( $MALS3^{lox/lox}; Pax3-Pro-cre$ ) [MMKO] (Li et al., 2000; Chang et al., 2004) (Fig. S5, D–F).

Levels of MALS-3 are differentially reduced in UBKO and MMKO mice (Fig. 9 C; Fig. S5, D–F). Importantly, gross abnormalities in kidney structure are only noted in the MMKO mice. Kidneys from MMKO mice are reduced in size (Fig. 9 D) with renal pathologies identical to  $MALS3^{+/+}$  mice (Fig. 9 E). These abnormalities are not observed in the UBKO mice (Fig. 9, D and E). Finally, in MMKO proximal tubules, but not UBKO, the CRB-3 and DLG complexes are affected (Fig. 9 F and unpublished data) in a manner similar to  $MALS3^{+/+}$ .

**Kidney organogenesis in MMKO**

Kidneys from adult MMKO mice phenocopy those of  $MALS3^{+/+}$  mice, indicating a shared developmental defect. Like the  $MALS3^{+/+}$ , kidneys from MMKO embryos (E14.5) show extensive apoptosis underlying the renal capsule (Fig. 10 A).



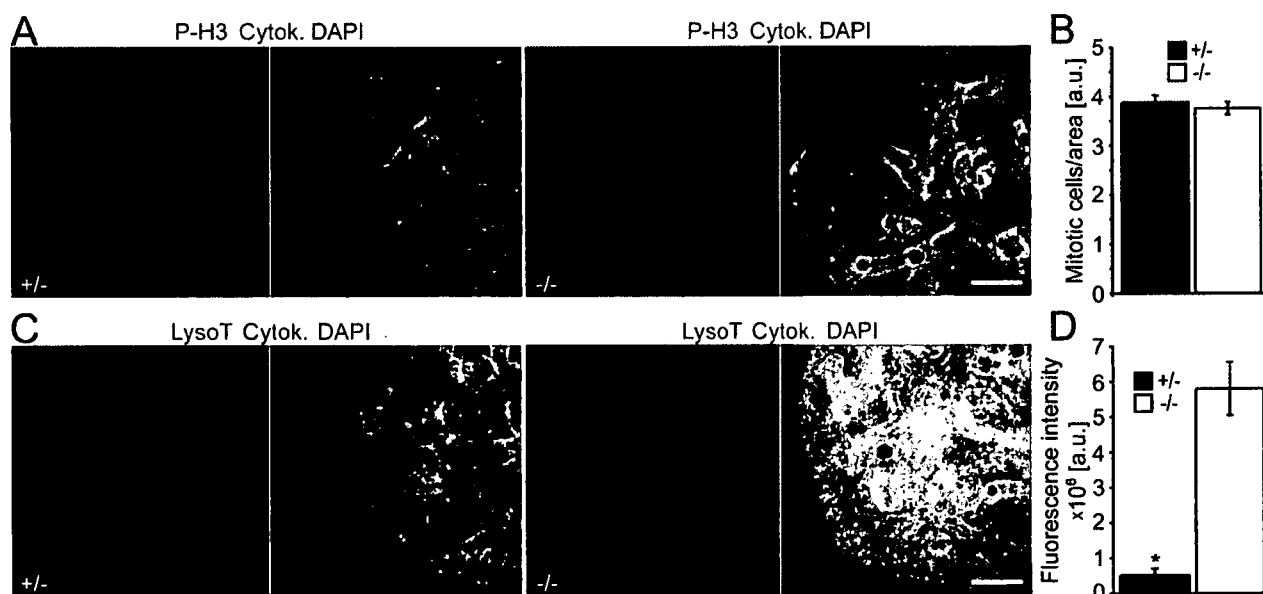
**Figure 7. Cilia show no changes in structure or localization for CRB-3.** (A) Cilia were visualized using anti-acetylated tubulin antibody. No obvious changes in cilia morphology were observed between control and *MALS-3<sup>g/g</sup>* kidneys. (B) CRB-3 (red) localizes to cilia in both control and *MALS-3<sup>g/g</sup>* kidneys. (C) Alignment of the intracellular portion of the translated protein sequences of canonical CRB-3 with three predicted protein sequences from the EST database. Underlined is the PDZ ligand in CRB-3 that is required for PALS/PATJ association; italicized are differences in the alternatively spliced C termini.

To determine whether defects in the CRB-3 and DLG polarity complexes underlie these developmental abnormalities, we examined the expression levels of these protein complexes in kidneys from MMKO embryos. Western blotting showed that both the CRB and DLG complexes were dramatically reduced (Fig. 10 B). These results show that *MALS-3* is a critical component of the CRB and DLG complexes and that disruption

of these complexes in the metanephric mesenchyme is associated with the renal defects in *MALS-3<sup>g/g</sup>* mice.

### Discussion

This study shows that *MALS-3* is essential for polarization of renal epithelia and for kidney development. Kidneys from



**Figure 8. Increased apoptosis in developing kidney of *MALS-3<sup>g/g</sup>*.** (A) Kidneys from E14.5 embryos were stained with anti-phospho-histone3 antibody (red) to visualize cells in mitosis and counterstained with cytokeratin (green) and DAPI (blue). No changes in cell proliferation were observed between *MALS-3<sup>g/g</sup>* and *MALS-3<sup>g/g</sup>* kidneys. (B) Quantification of the number of mitotic cells. (C) LysoTracker (red) was used to label apoptotic cells and counterstained with cytokeratin (green) and DAPI (blue) in embryonic kidneys (E14.5). *MALS-3<sup>g/g</sup>* kidneys showed a dramatic increase in the number of apoptotic cells. (D) Quantification of LysoTracker fluorescence intensity from kidneys. Bar  $\square$  100  $\mu$ m.



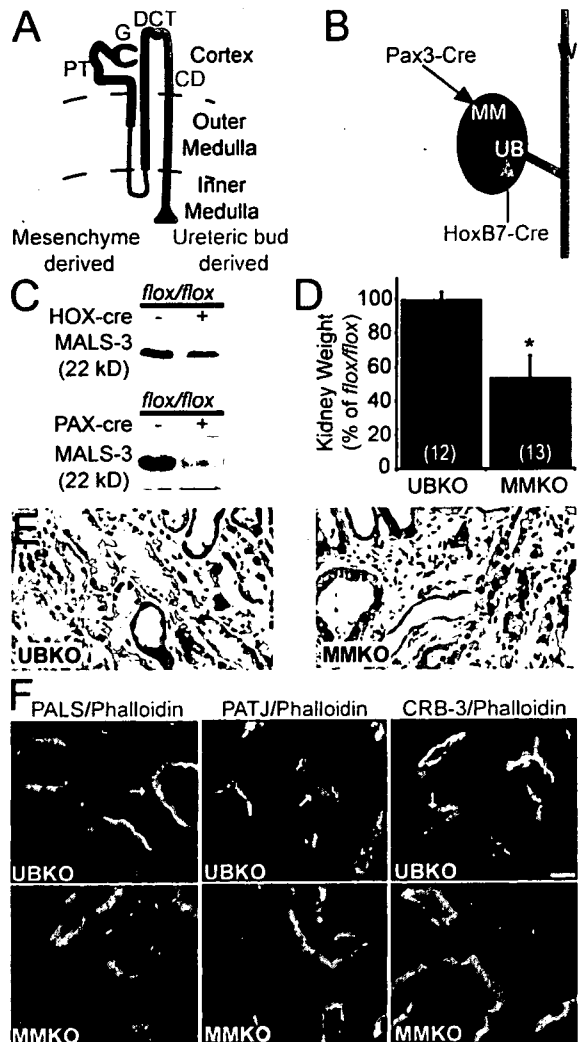
*MALS-3<sup>fl/fl</sup>* mice display tubule dysgenesis, interstitial fibrosis, and cysts. These pathologies arise from defective polarization of tubule epithelia derived from the metanephric mesenchyme. MALS-3 mediates polarization of tubular epithelia through interactions with the CRB and DLG polarity complexes. Loss of MALS-3 disrupts polarized expression of these two critical protein complexes, establishing MALS-3 as an essential component of each. Furthermore, these data demonstrate that primary abnormalities in epithelial polarization can cause renal dysgenesis, fibrosis, and cysts.

MALS-3 is a component of epithelial CRB-3 and DLG polarity complexes. *LIN-7* was originally identified in *C. elegans* as a gene essential for vulval development (Simske et al., 1996). In vulval precursor cells, LIN-7 is one component of a heterotrimeric PDZ protein complex that includes LIN-2 and LIN-10 (Kaech et al., 1998). Assembly of the complex is mediated through LIN-2, which binds LIN-7 through their complementary L27 domains, and binds LIN-10 through a more N-terminal site (Kaech et al., 1998). Vulval development requires signaling by an EGF-like receptor (LET-23) that binds the PDZ domain of LIN-7 (Simske et al., 1996). The LIN-2/-7/-10 complex localizes LET-23 to the basolateral surface where it binds its ligand (Kaech et al., 1998). Mutations in *lin-2*, *-7*, or *-10* mislocalize LET-23 to the apical domain and block vulval induction.

The LIN-2 (CASK)/LIN-7 (MALS)/LIN-10 (MINT-1) complex is conserved in mammalian brain, where it occurs both pre- and post-synaptically (Butz et al., 1998; Borg et al., 1999; Jo et al., 1999). Presynaptically, CASK binds to liprin-βs (Olsen et al., 2005a). Postsynaptically, the PDZ domain of MALS binds to the C-terminal tail of NMDA receptors (Jo et al., 1999). Altered neurotransmitter release in neurons lacking MALS is consistent with the MALS complex organizing the presynaptic active zone and vesicle cycling (Olsen et al., 2005a). Composition of the MALS complex in kidney differs fundamentally from that in *C. elegans* or brain, as kidney epithelium lacks MINT-1 (Okamoto and Sudhof, 1997). MINT-3 occurs in kidney, but it lacks the CASK interaction domain (Okamoto and Sudhof, 1997). MINT or liprin-β isoforms were not detected in MALS complexes isolated from kidney (Fig. 3).

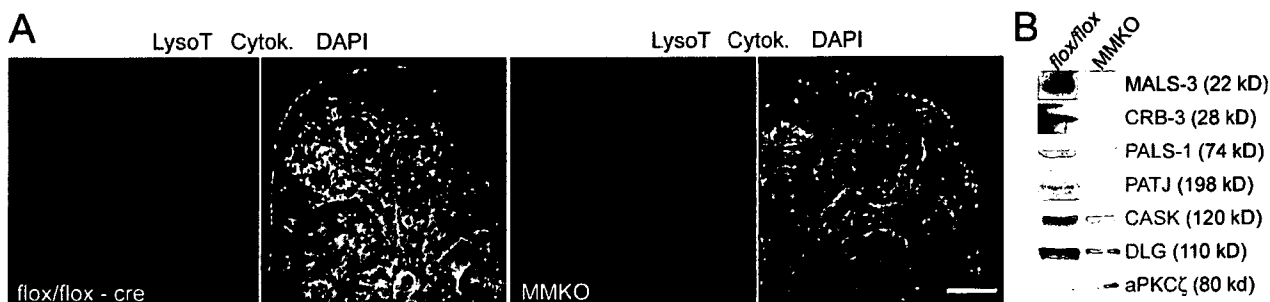
Our work shows that MALS-3 occurs in two conserved epithelial cell polarity complexes and differentially colocalizes with these complexes in specific renal cell populations. In proximal tubule epithelia, MALS localizes to the tight junction—likely owing to its association with the CRB complex. *crb* was the first epithelial polarity gene characterized in *Drosophila* (Tepass et al., 1990). It is a transmembrane protein whose C-terminal tail interacts with the PDZ domain of Stardust, SDT (PALS) (Roh et al., 2002), or dPATJ (PATJ) (Bhat et al., 1999). SDT is a membrane-associated guanylate kinase (MAGUK) that contains two L27 protein motifs (Kamberov et al., 2000). The first and second L27 domains of SDT bind to the L27 domains of dPATJ (Roh et al., 2002) and MALS (Kamberov et al., 2000), respectively.

Additionally, MALS associates with the basolateral DLG complex. In invertebrates, DLG mediates apico-basal polarization



**Figure 9. Defective polarity cues in renal mesenchyme lacking MALS-3.** (A) Cartoon shows nephron segments and their embryonic origins. Abbreviations are as in Fig. 2 A. (B) Cartoon shows the expression of cre recombinase (cre) driven by *Pax3* promoter (*Pax3-cre*; blue) or *HoxB7* promoter (*HoxB7-cre*; orange). MM, metanephric mesenchyme; UB, ureteric bud; W, Wolffian duct. (C) Western blotting of adult kidney homogenates from *flox/flox* mice shows differential loss of MALS-3 in mice expressing cre from the *HoxB7* (*Hox-cre*) and *Pax3* (*Pax-cre*) promoters. (D) Elimination of MALS-3 from the metanephric mesenchyme fully reproduces the *MALS-3<sup>fl/fl</sup>* kidney deficiencies. *MALS-3 flox/flox; Pax3-Pro-cre* (MMKO) mice have kidneys that are reduced in magnitude to similar extent as *MALS-3<sup>fl/fl</sup>* mice (54.7% ± 13.3;  $P < 0.01$ ). In contrast, kidneys from *MALS-3 flox/flox; HoxB7-Pro-cre* (UBKO) mice have kidneys similar in size (99.7% ± 4.6) to littermate controls. (E) Trichome-stained kidney section from a 6-wk-old UBKO mouse shows normal renal anatomy (left), whereas kidney from a MMKO mouse (right) shares anatomical abnormalities with *MALS-3<sup>fl/fl</sup>* mice, including tubular dilatation and fibrosis (arrow). (F) Immunohistochemistry from the cortex of kidneys from 6-wk-old UBKO and MMKO mice stained with phalloidin (green) to identify proximal tubule brush borders and DAPI (blue) for nuclei. PALS, PATJ, and CRB-3 all properly localize to the tight junction (arrows) of epithelia in proximal tubules of a kidney from UBKO mouse (F, top). In contrast, PALS, PATJ, and CRB-3 are lost from tight junction in proximal tubules from a MMKO mouse (F, bottom). Asterisk marks apical localization of CRB-3 in collecting duct.

of epithelia and controls imaginal disc proliferation (Woods and Bryant, 1991). DLG is a MAGUK containing a single L27 domain that binds to the Arst of two L27 domains in CASK



**Figure 10. Increased apoptosis associated with disrupted polarity complexes in developing kidney of MMKO.** (A) Apoptotic cells were visualized in embryonic kidneys (E14.5) with LysoTracker (red). Cytokeratin (green) and DAPI (blue) label epithelia and nuclei, respectively. As compared with control littermates, MMKO kidneys showed a dramatic increase in the number of apoptotic cells. Bar = 100  $\mu$ m. (B) Kidney homogenates from *flox/flox* and MMKO mice immunoblotted for proteins of the CRB-3 and DLG polarity complexes show reduced expression of both complexes in MMKO embryonic kidneys.

(Lee et al., 2002), whose second L27 domain interacts with MALS (Straight et al., 2000). Other than CRB, all of these polarity proteins contain PDZ domains, whose binding surfaces are largely uninvolved in assembly of these protein complexes and may recruit additional components. MALS is the only known protein that functions as a core component of both the CRB and DLG complexes, positioning MALS as a central regulator of these complexes and cell polarity.

L27 domain interactions mediate assembly of MALS with the CRB and DLG complexes. Recently, the L27 domain structures of the DLG/CASK and PALS/PATJ complexes were independently determined by NMR and X-ray crystallography (Feng et al., 2004; Li et al., 2004). Each L27 domain comprises three  $\alpha$ -helices, and heterodimers form from a four-helical bundle of the Arst two helices. In the NMR structure of DLG/CASK, the third helix of each L27 formed another four helical bundles, assembling a tetramer (Feng et al., 2004). In contrast, X-ray structure showed only the hydrophobic residues of the PATJ L27 domains were necessary for assembling the PALS/PATJ tetramer (Li et al., 2004). Additional NMR studies showed that PALS/PATJ forms a tetrameric structure that resembles CASK/DLG and uses the C-terminal half of the third  $\alpha$ -helix of all four L27 domains (Feng et al., 2005).

These structural studies used two isolated L27 domains. However, many proteins, including CASK and PALS, have tandem L27 domains. We show that stability of tandem L27 structure requires occupation of both domains, which results in the formation of ternary complexes containing MALS/CASK/DLG or MALS/PALS/PATJ with a 1:1:1 stoichiometry. In each complex, four different L27 domains form a cooperative tetrameric complex. Presumably, the third  $\alpha$ -helices in the L27 domains interact between tandem L27 pairs, imparting stability and blocking formation of higher order oligomers. Future structural analyses will determine precise assembly mechanisms for these ternary complexes. Most importantly, these data reveal a mechanism for assembly of the CRB and DLG complexes and explain their dissolution in *MALS-3<sup>Δ/Δ</sup>* mice.

MALS-3 polarizes the apical DLG and basolateral DLG complexes

Loss of the CRB complex from proximal tubule tight junctions in *MALS-3* mutants suggests that MALS-3, through its interaction

with PALS (Kamberov et al., 2000), assembles the tight junction complex. Our results are supported by studies in MDCK cells that show MALS-3 knockdown disrupts the localization of PALS and PATJ (Straight et al., 2006). The current study makes several important advances on the role of MALS-3 in regulation of the CRB-3 complex. First, we show that the loss of PALS and PATJ in proximal tubule epithelia lacking MALS-3 is accompanied by a specific loss of CRB-3 from the tight junction. Importantly, these changes are not conserved in collecting duct epithelia, from which MDCK cells derive. This may explain why previous studies of MALS in MDCK cells have not described changes in CRB-3 expression, localization, or function (Straight et al., 2006). We also show that disruption of the CRB-3 complex results from the instability of the unbound PALS L27C domain, but direct interactions between MALS-3 and PATJ or CRB-3 cannot be ruled out. Finally, we show that CRB-3 properly localizes to cilia in these *MALS-3*-deficient epithelia. An alternatively spliced CRB-3 variant, CRB-3b that lacks the PDZ ligand, likely accounts for these discrete localizations at the tight junction and cilia.

In addition to regulating assembly of the CRB complex, MALS-3 directs basolateral localization of DLG. The molecular mechanism underlying DLG mislocalization remains uncertain. MALS-3 associates with DLG through the MAGUK scaffolding protein CASK (Lee et al., 2002; Leonoudakis et al., 2004), suggesting that MALS-3 may directly control basolateral expression of DLG. In support of this, DLG and CASK coimmunoprecipitate with MALS-3 from kidney homogenates. Alternatively, DLG/LGL complexes that cross the apico-basal boundary may be recruited to aPKC by MALS-3. This recruitment may result in phosphorylation of LGL and restriction of the DLG/LGL complex to the basolateral membrane. Alternatively, the CRB complex may be required to apically exclude DLG. Fitting with this model, overexpression of CRB can rescue DLG polarization in *C. elegans* (Segbert et al., 2004).

Polarity defects in *MALS-3<sup>Δ/Δ</sup>* are restricted to specific renal epithelia

Why does MALS-3 differentially polarize in distinct epithelial cell populations? In proximal tubule epithelia derived from metanephric mesenchyme, MALS-3 is expressed at the tight junction and basolateral membrane, whereas in cells derived

from ureteric bud, MALS-3 is exclusively detected along the basolateral membrane. In *Drosophila*, cell-specific mechanisms have been identified for the MALS homologue, DLIN-7 (Bachmann et al., 2004). Here, the PALS homologue (SDT) associates with DLIN-7 at epithelial junctions, whereas *Drosophila* DLG interacts with DLIN-7 at the neuromuscular junction (Bachmann et al., 2004). Ectopic expression of SDT or DLG mediates DLIN-7 delivery to epithelial and neuromuscular junctions, respectively. This suggested that junctional targeting of MALS is downstream of both SDT and DLG (Bachmann et al., 2004). Our results are not consistent with this model. Loss of MALS-3 in proximal tubule epithelia causes dissolution of the CRB complex and redistribution of DLG, demonstrating that MALS are not always downstream of either PALS or DLG. Instead, MALS may primarily determine polarization in specific cell populations. In such a model, the PDZ binding partner for MALS could serve as the tight junction anchor. Identification of physiological PDZ ligands for MALS may help understand its cell type-specific functions.

Does MALS determine polarity in tissues other than kidney? In *Drosophila* with mutations to components of the CRB complex, numerous ectodermally derived epithelia are disorganized and degenerated (Tepass et al., 2001). The viability and fertility of *MALS-3* knockout mice indicate that many epithelial cell populations develop and function properly without MALS-3. The additional MALS isoforms may serve redundant functions; indeed, *MALS*-deficient mice die from respiratory failure (Olsen et al., 2005a). That these mice show grossly normal size and morphology (Olsen et al., 2005a) suggests that MALS are not essential for polarizing all cell populations. Future studies of *MALS* triple-knockout mice should help define which cell types require MALS for polarization and determine how other pathways compensate or work in parallel.

#### Implications for renal development and disease

Our studies reveal that renal defects arise solely from a loss of MALS-3 in epithelia derived from metanephric mesenchyme. MALS-3 is also present in epithelial cells derived from ureteric bud (Olsen et al., 2005b), but restricted ablation of MALS-3 in the ureteric bud neither causes changes in renal morphology nor disrupts CRB-3 polarization. By contrast, loss of MALS-3 in the metanephric mesenchyme is sufficient to recapitulate the renal pathologies observed in *MALS-3<sup>fl/fl</sup>* mice. Localization of MALS-3 to the tight junctions in renal epithelia of mesenchymal origin suggests that tight junction-associated MALS-3 underlies the renal defects observed in *MALS-3<sup>fl/fl</sup>*.

Mesenchymal to epithelial transition (MET) is a critical process underlying the development of numerous organs, including the kidney. Differentiation of epithelia from mesenchyme requires the establishment cell polarity. In MMKO mice, polarization cues originating from CRB and DLG complexes are disrupted. This likely leads to changes in the efficacy of epithelial cell polarization during MET, resulting in apoptosis and smaller kidneys in *MALS-3* mutants. However, most renal epithelia in *MALS-3<sup>fl/fl</sup>* show normal cytoarchitecture. This indicates that redundant or parallel pathways compensate, albeit incompletely,

for loss of the CRB and DLG complexes. Further analysis of *MALS-3<sup>fl/fl</sup>* embryos will be useful for determining the developmental processes regulated by these polarity complexes.

The converse of MET may underlie renal tubule simplification/dedifferentiation and Abrosis in *MALS-3* mutants. Recent studies suggest that epithelial to mesenchymal transition (EMT) of tubule epithelia is a significant source of disease-related Abroblasts (Kalluri and Neilson, 2003; J.M. Lee et al., 2006). A critical step in EMT is a loss of cell polarity. Because *MALS-3* epithelia are improperly polarized, uncontrolled EMT could account for the dedifferentiated tubular epithelia and Abrosis in *MALS-3* mutants. Alternatively, renal epithelia lacking MALS-3 may be increasingly susceptible to external cues, such as injury or inflammation, which can trigger EMT (Kalluri and Neilson, 2003; J.M. Lee et al., 2006). As defects in epithelial polarization and associated MET/EMT play central roles in renal cysts and Abrosis, *MALS-3<sup>fl/fl</sup>* mice should provide an important new model for understanding and treating these common kidney diseases.

## Materials and methods

### Antibodies

Isoform specific and pan antibodies against MALS-1, -2, and -3 were generated in rabbits as described previously (Misawa et al., 2001). Anti-SAP-97 antibody was generated in rabbit (Topinka and Bredt, 1998); for immunohistochemistry an anti-SAP-97 antibody from Morgan Sheng (Massachusetts Institute of Technology, Cambridge, MA) was used. Rabbit anti-JAMSA and anti-claudin antibodies were purchased from Invitrogen. PALS, PATJ, and Crumbs-3 antibodies were gifts from Ben Margolis (University of Michigan, Ann Arbor, MI) and rabbit anti-LGL antibody was provided by Valeri Vasioukhin (Fred Hutchinson Cancer Research Center, Seattle, WA). Rabbit anti-PAX2 was from ABCO. Goat anti-aquaporin-2 antibody and rabbit anti-aPKC $\beta$  were from Santa Cruz Biotechnology, Inc. Rabbit anti-PAR-3 and anti-phospho-histone3 antibodies were from Upstate Biotechnology and goat anti-scribble antibody was from GeneTex, Inc. Mouse anti-CASK, anti-ZO-2, anti- $\beta$ -catenin, anti-E-cadherin, and anti-N-cadherin were all purchased from Transduction Laboratories. Alexa-conjugated Phalloidin and secondary antibodies were purchased from Molecular Probes and mouse anti-pancytokeratin was from Sigma-Aldrich.

### Mouse lines

The following mouse lines were used in this study: STOCK G1(ROSA)26-Sortm1(Smo/EYFP)Amc/J, STOCK Tg(HoxB7-cre)13Amc/J, 129S4/SvJaeSor-G1(ROSA)26Sortm1(FLP1)Dym/J, C57BL/6, and 129/Sv were purchased from The Jackson Laboratory. Pax3-Pro-Cre mice were a gift from Jonathan Epstein (University of Pennsylvania, Philadelphia, PA). *MALS-1*, -2, and -3 have been described previously (Misawa et al., 2001; Olsen et al., 2005a). For characterization of mice lacking MALS-1 and -2, double-knockout mice were compared with wild-type mice of a similar age and genetic background. Generation of *MALS-3* flox mice is described below.

### Isolation of MALS-3 genomic DNA and construction of targeting vector

Isolation of MALS-3 genomic DNA (BAC clone, mCG15974) has been described previously (Olsen et al., 2005a). For construction of the targeting vector, a 1.9-kb region encoding the targeted exons (the fourth and fifth exon) of MALS-3 was PCR-amplified, digested with ClaI, and subcloned into the ClaI site of pks2loxPFRNT (a gift from Shinya Yamataka, Kyoto University, Kyoto, Japan). A 1-kb region downstream from the targeted exons was PCR-amplified, digested with BamHI and EcoRI, and subcloned into the BamHI-EcoRI sites of pks2loxPFRNT. Finally, a 5.8-kb genomic region upstream to the targeted exons was PCR-amplified, digested with KpnI and BamHI, and inserted into the KpnI-Sall sites of the pks2loxPFRNT vector. Two loxP sites flank targeted exons and a neomycin cassette. Two PRT sites flank the neomycin cassette for its removal.

### Generation of MALS-3 loxP mice

The targeting vector was linearized and electroporated into R-1 ES cells. Clones resistant to G418 and gancyclovir were analyzed for recombination by PCR. To ensure proper homologous recombination, PCR-positive

clones were further analyzed by Southern blotting using probes containing genomic sequences outside of the targeting vector and with a neo probe. Properly targeted clones were injected into blastocysts from C57BL6 mice and transferred to surrogate mothers (Transgenic Facility, Stanford University, Stanford, CA). Male chimeras were mated with 129S4/SvJae-Sor-GI(ROSA)26Sortm1(FLP1)Dym/J (The Jackson Laboratory) females for transmission of the mutated allele through the germ line and for removal of the neomycin cassette from the targeted allele. Genotypes for *MALS-3<sup>loxP</sup>* mice lacking the neomycin cassette were determined by Southern blotting or by PCR using the primers: 5'GAAAATGCTTCTGTCGGTTTGC-3' and 5'ATTGCTGTCACCTGGTGGTGGC-3' which yields a 280-bp product for the wild-type allele and a 350-bp product for the targeted allele. Presence of the *cre*-recombinase and EYFP transgenes was determined using the following primer pairs: 5'GAAAATGCTTCTGTCGGTTTGC-3' and 5'ATTGCTGTCACCTGGTGGTGGC-3' for *cre* and 5'CCCTGAAGTTCATCTGCCACC-3' and 5'GGACTTGTACAGCTCGTCCATGCC-3' for EYFP.

#### Protein expression and purification

Genes corresponding to the L27 domain of rat DLG (L27<sup>DLG</sup>, residues 1–65), the L27 domain of mouse MALS (L27<sup>MALS</sup>, residues 2–78), and the tandem L27 domain of rat CASK (L27<sup>NL27CASK</sup>, residues 329–460) were PCR amplified from the respective full-length cDNAs. The single-chain fusion protein, containing L27<sup>DLG</sup>, L27<sup>NL27CASK</sup>, and L27<sup>MALS</sup> connected with a thrombin-cleavable segment (Leu-Val-Pro-Arg-Gly-Ser-Ser-Gly), was cloned into a modified version of the pET32a vector in which the S-tag and the thrombin recognition site were replaced by a sequence encoding a protease 3C cleavage site (Leu-Glu-Val-Leu-Phe-Gln-Gly-Pro). Similarly, the single chain fusion protein, containing L27<sup>DLG</sup> and L27<sup>NL27CASK</sup> connected with a thrombin-cleavable segment, was PCR-amplified and inserted into the modified pET32a vector. Bacterial cells harboring the fusion protein expression plasmid were grown at 37°C, and protein expression was induced by IPTG at the same temperature for 3 h. The His-tagged, thioredoxin-containing protein was purified under native conditions using Ni-NTA agarose (QIAGEN) affinity chromatography. After protease 3C digestion the N-terminal His-tag and thioredoxin were removed by passing the digestion mixture through an S-200 gel filtration column, then the single-chain protein was digested by thrombin and the L27 domain complex protein was further passed through the same size-exclusion column. The expression and purification of L27<sup>MALS</sup>/L27<sup>CASK</sup> and L27<sup>NL27CASK</sup>/L27<sup>DLG</sup> complex proteins were described previously (Feng et al., 2004, 2005).

#### Kidney immunohistochemistry

Adult mice were anesthetized and perfused with 2% paraformaldehyde in PBS. Kidneys were removed, immersed in the same fixative for 2 h at 4°C, and then cryoprotected in PBS containing 30% sucrose overnight at 4°C. Frozen sections (10 µm) were rehydrated with PBS containing 0.1% Triton X-100 (PBS-X) for 20 min and incubated for 1 h in blocking solution (PBS-X containing 1% BSA). Primary and secondary antibodies were diluted in blocking solution and incubated overnight at 4°C and 2 h at room temperature, respectively. Antibody incubations were followed by three washes in PBS-X. Sections were mounted with coverslips in Fluoromount-G (Southern Biotechnology Associates, Inc.). Images were taken with a confocal microscope (LSM 5 Pascal Axioplan 2; Carl Zeiss Microimaging, Inc.) under Zeiss 63× 1.4 or 40× 1.3 oil immersion objectives or a Zeiss 10× 0.3 objective at room temperature and acquired using LSM 5 Pascal (v.3.2) software. Images were processed using Adobe Photoshop. To study primary molecular defects in *MALS-3<sup>loxP</sup>* mice, only kidneys that showed normal arrangement of tubules with mild anatomical abnormalities were used for localizing aPKC/Crumbs/DLG complexes. More than three sections from six KO and six control littermates were examined. Proximal tubules were identified by phalloidin staining of apical brush border, whereas collecting ducts were stained using aquaporin-2 antibody.

For hematoxylin and eosin or trichrome staining, perfused kidneys were immersed in 4% paraformaldehyde in PBS overnight at 4°C, dehydrated using an alcohol series and xylene, and embedded in paraffin. Paraffin sections (4 µm) were cut on a microtome and mounted onto slides. All light images were taken using an AxioCam HRC camera mounted to a microscope (Eclipse E6600; Nikon) with 10× 0.3 and 20× 0.5 objectives at room temperature. Images were acquired with AxioVision (v4.6) software and processed in Adobe Photoshop.

#### Renal function

Mice were housed in metabolic cages on a 12-h light-dark cycle and fed ad libitum on normal chow. After 24 h, urine was collected under oil, animals were anesthetized with Inactin (100 mg/kg, i.p.), and blood was collected by cardiac puncture. Blood and urine chemistries were measured by

automated methods (IDEXX, West Sacramento, CA). For measurements of urine diluting and concentrating ability, urine osmolarity was measured using a vapor pressure osmometer (Wescor) after animals were fed a high water content diet (Bouby et al., 1990, 1996) for 48 h and then again after mice (12 h) were injected with deamino-Cys, D-Arg<sup>9</sup>-vasopressin (DDAVP, 1 µg/kg, i.p.) and feeding water was withdrawn.

#### Immunoprecipitations

Adult mouse kidneys from *MALS-3<sup>loxP</sup>* or *MALS-3<sup>+/+</sup>* mice were homogenized in three volumes of STE buffer (320 mM sucrose, 20 mM Tris, pH 7.4, and 2 mM EDTA) containing 10 µg/ml leupeptin, 10 µg/ml aprotinin and 200 µg/ml PMSF. Homogenates were centrifuged at 20,000 g for 1 h and pellets were resuspended in TET buffer (20 mM Tris, pH 8.0), 1 mM EDTA, and 1.3% Triton X-100) containing 10 µg/ml leupeptin, 10 µg/ml aprotinin, and 50 µg/ml PMSF. Lysates were pelleted at 100,000 g for 1 h. Precleared lysates were immunoprecipitated with 5 µg of *MALS-3* antibody or control rabbit IgG overnight at 4°C. To collect immunoprecipitated protein complexes, 80 µl of a 50% protein A-Sepharose slurry was added to the lysates and incubated for 1 h at 4°C. Immunoprecipitates were washed extensively and loaded onto SDS-PAGE to separate the proteins. Gels were either silver stained or transferred to nitrocellulose for Western blotting.

#### Nano-LC-ESI-Qq-TOF tandem mass spectrometry analysis

Gel bands were reduced with 10 mM dithiothreitol (DTT) followed by alkylation with 55 mM iodoacetamide. Proteins were digested with trypsin and extracted with a 50% acetonitrile/5% formic acid solution. The peptides were dried down and resuspended in 0.1% formic acid then separated via HPLC using a 75 µm × 15 cm reverse-phase C-18 column (LC Packings) running a 3–32% acetonitrile gradient in 0.1% formic acid on an Agilent 1100 series HPLC. The LC eluent was coupled to a micro-spray source attached to a QSTAR Pulsar mass spectrometer (MDS Sciex). Peptides were analyzed in positive ion mode.

#### Analytical gel filtration

Size-exclusion chromatography was performed on an AKTA FPLC system using a Superose 12 10/30 column (GE Healthcare). Protein samples were dissolved in 100 mM potassium phosphate buffer containing 1 mM DTT. The column was calibrated with the low molecular mass column calibration kit from GE Healthcare.

#### Urea denaturation

Urea denaturation of L27 domain complexes was monitored by acquiring circular dichroism spectra of protein samples at each urea concentration. Data were collected on a JASCO J-720 spectropolarimeter at room temperature. The sample contained 15 µM protein in buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 1 mM DTT).

#### Cell death and proliferation

To detect dying cells, LysoTracker (Invitrogen) labeling of embryonic kidneys was performed as described previously (Grieshammer et al., 2005). In brief, isolated kidneys (six kidneys each for control and *MALS-3<sup>loxP</sup>*) were incubated with 5 µl/ml LysoTracker solution in HBSS at 37°C for 30 min, rinsed in HBSS, fixed in 4% PFA in PBS, and stored at 4°C in 100% methanol. After rehydration into PBS, E14.5 kidneys were sectioned (100 µm), permeabilized in PBS containing 0.1% Tween 20, and stained with anti-cytokeratin (Sigma-Aldrich). After extensive washing, cytokeratin antibody was detected using Alexa488-conjugated donkey anti-mouse antibody (Invitrogen) and nuclei were labeled with DAPI. All images were taken with a confocal microscope (LSM 5 Pascal Axioplan 2; Carl Zeiss Microimaging, Inc.) under Zeiss 25× 0.8 objective (zoom setting 1 or 2) at room temperature and acquired using LSM 5 Pascal (v.3.2) software. Total fluorescent intensity was analyzed using MetaMorph software and images were processed using Adobe Photoshop. The average intensity of three individual optical sections from each stack was calculated. Five stacks of individual kidneys were analyzed for each condition. For apoptosis in E12.5 whole mounts, kidneys were rehydrated into PBS containing Tween 20 (0.1%) and counterstained with anti-PAX2 antibody. The rest of the staining procedure was performed as described above. To detect cells in mitosis, isolated kidneys (13 sections from 5 control kidneys and 14 from 5 *MALS-3<sup>loxP</sup>* kidneys) were fixed in 4% PFA, washed, sectioned (100 µm), and incubated with rabbit anti-phospho-histone H3 antibody (1:200; Upstate Biotechnology) and co-stained with anti-cytokeratin as above. Alexa546-conjugated donkey anti-rabbit antibody was used for labeling phospho-histone antibody. All images were obtained with a confocal microscope and >20 optical sections were analyzed for each kidney.