Modulatory calcineurin-interacting proteins 1 and 2 function as calcineurin facilitators *in vivo*

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The calcium-activated phosphatase calcineurin is regulated by a binding cofactor known as modulatory calcineurin-interacting protein (MCIP) in yeast up through mammals. The physiologic function of MCIP remains an area of ongoing investigation, because both positive and negative calcineurin regulatory effects have been reported. Here we disrupted the *mcip1* and *mcip2* genes in the mouse and provide multiple lines of evidence that endogenous MCIP functions as a calcineurin facilitator *in vivo*. Mouse embryonic fibroblasts deficient in both *mcip1/2* showed impaired activation of nuclear factor of activated T cells (NFAT), suggesting that MCIP is required for efficient calcineurin–NFAT coupling. Mice deficient in *mcip1/2* showed a dramatic impairment in cardiac hypertrophy induced by pressure overload, neuroendocrine stimulation, or exercise, similar to mice lacking *calcineurin Aβ*. Moreover, simultaneous deletion of *calcineurin Aβ* in the *mcip1/2*-null background did not rescue impaired hypertrophic growth after pressure overload. Slow/oxidative fiber-type switching in skeletal muscle after exercise stimulation was also impaired in *mcip1/2* mice, similar to *calcineurin Aβ*-null mice. Moreover, CD4$^+$ T cells from *mcip1/2*-null mice showed enhanced apoptosis that was further increased by loss of *calcineurin Aβ*. Finally, *mcip1/2*-null mice displayed a neurologic phenotype that was similar to *calcineurin Aβ*-null mice, such as increased locomotor activity and impaired working memory. Thus, a loss-of-function analysis suggests that MCIPs serve either a permissive or facilitative function for calcineurin–NFAT signaling *in vivo*.

heart | PP2B | signal transduction

Calcineurin is a serine/threonine-specific protein phosphatase that is uniquely activated by sustained elevations in intracellular calcium (1, 2). Calcineurin is composed of a 59- to 63-kDa catalytic subunit [calcineurin A (CnA)] and a 19-kDa calcium-binding subunit [calcineurin B (CnB)]. Three mammalian *CnA* catalytic genes (α, β, and γ) and two *CnB* regulatory genes (*B1* and *B2*) have been identified in vertebrates. Once activated, calcineurin directly dephosphorylates nuclear factor of activated T cells (NFAT) transcription factors within the cytoplasm, promoting their translocation into the nucleus (1). Calcineurin–NFAT signaling is critically involved in regulating the cardiac hypertrophic growth response and cellular protection after ischemia in the heart (3–5). In T cells, calcineurin–NFAT signaling regulates cytokine gene expression and cellular activation after antigen challenge (1, 6). Indeed, genetic deletion of CnAβ leads to severe defects in T cell maturation and activation (7). In skeletal muscle, calcineurin promotes cellular differentiation and controls fiber-type switching between a slow/oxidative and a fast/glycolytic program (8–10). In the central nervous system, calcineurin–NFAT signaling regulates different aspects of brain function such as synaptic plasticity, neurotransmission, activity-dependent gene expression, and memory (11–14). For example, forebrain-specific deletion of calcineurin increased locomotor activity and reduced specific aspects of memory function (11, 12).

Although calmodulin complexed with Ca^{2+} is the only known activator of calcineurin, a wide range of inhibitory proteins has been described. One such inhibitor, known as modulatory calcineurin-interacting protein (MCIP), or Down Syndrome Critical Region 1 (DSCR1) and DSCR1-like proteins/calcipressins, is composed of a gene family that includes *mcip1*, *mcip2*, and *mcip3* (15). When overexpressed, MCIPs inhibit calcineurin activity both *in vitro* and *in vivo* by direct binding to the active site, hence blocking NFAT activation (16). However, in yeast and *Cryptococcus neoformans*, the MCIP homologue, RCN1/CBP1, has functional and phenotypic characteristics of a calcineurin activator (17–19). Consistent with this observation, *mcip1*$-/-$ mice showed an impaired cardiac hypertrophic response to pressure overload, suggesting that it may also function as a calcineurin facilitator *in vivo* (20). However, under other conditions or in other mammalian cell types, endogenous MCIP can mediate an inhibitory function (21).

Results

Generation and Characterization of *mcip1*$-/-$ and *mcip2*$-/-$ Mice. Many studies conducted in mammalian model systems have concluded that MCIPs function as negative regulators of calcineurin activity. However, some experiments in lower organisms have suggested that these proteins might also selectively augment calcineurin signaling (15, 17, 18). To elucidate the function of MCIP proteins *in vivo* the genes encoding *mcip1* and *mcip2* were targeted by homologous recombination in embryonic stem cells. Exons 5 and 6 were deleted from *mcip1*, whereas exons 2 and 3 were deleted from *mcip2* (Fig. 1 *A* and *B*). *mcip1*$-/-$ and *mcip2*$-/-$, as well as *mcip1/2* double-null mice (2KO) mice, were viable and able to breed as adults. However, *mcip1*$-/-$ mice demonstrated a $\approx$10% reduction in body weight as adults and a reduction in fertility as they aged (data not shown). Protein extracts were generated from heart, skeletal muscle, thymus, and brain for Western blotting with MCIP1-, MCIP2-, and MCIP3-specific polyclonal antisera from WT and 2KO mice (Fig. 1*C*). The data demonstrate that MCIP1 and MCIP2 protein is present in heart, skeletal muscle, and brain of WT mice, but not in 2KO mice. MCIP3 protein was not detected in heart and skeletal muscle. Surprisingly, the brain expresses the most MCIP1/2 protein, in contrast to reported levels of mRNA distribution (22, 23). There was no compensatory alteration in CnA or CnB protein levels in the absence of *mcip1* and *mcip2* (Fig. 1*C* and data not shown).

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Abbreviations: 2KO, *mcip1/2* double-null mice; 3KO, *mcip1/2* $\times$ *CnA*β triple-null mice; Ang II, angiotensin II; CnA, calcineurin A subunit; CnB, calcineurin B subunit; HW/TL, heart weight/tibia length; IL-2, interleukin-2; MCIP, modulatory calcineurin-interacting protein; MEF, mouse embryonic fibroblast; NFAT, nuclear factor of activated T cells; TAC, transverse aortic constriction; PP1, protein phosphatase 1.

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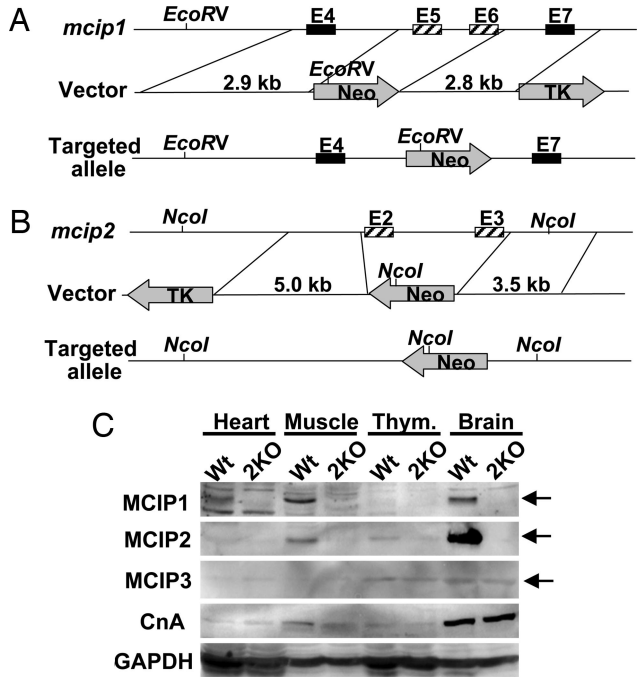

Fig. 1. Generation of *mcip1* and *mcip2* gene-targeted mice. (*A* and *B*) Schematic representation of the *mcip1* and *mcip2* genetic loci and the exons that were targeted for replacement with neomycin (Neo) in embryonic stem cells. The position of the relevant restriction enzymes used for Southern blotting is shown. (*C*) Western blotting for MCIP1, MCIP2, MCIP3, CnA, and GAPDH protein from the indicated tissues of WT and 2KO mice. The arrow shows the position of MCIP1.1/2/3.

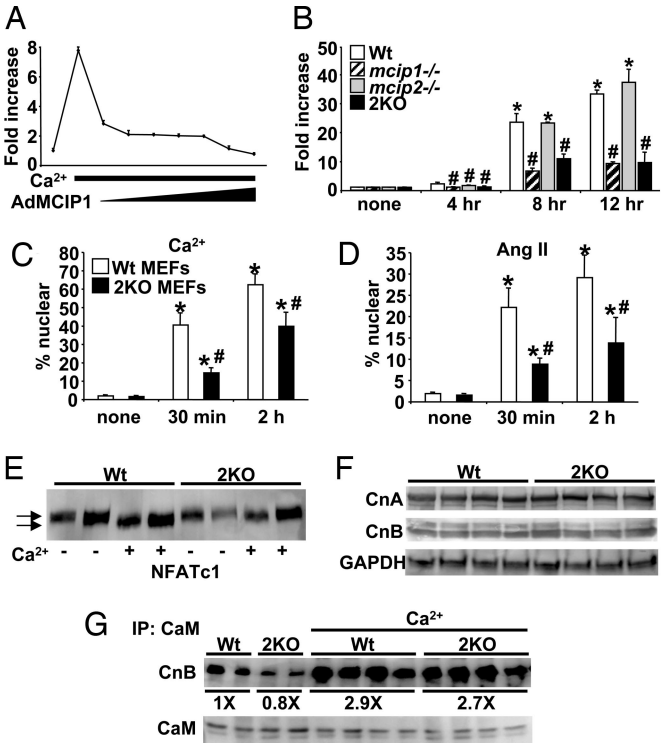

Fig. 2. Assessment of calcineurin function in 2KO MEFs. (*A*) NFAT transcriptional activity in WT MEFs infected with the NFAT-dependent luciferase reporter adenovirus and increasing concentration of AdMCIP1 at baseline or in response to thapsigargin (2 μM) for 4 h. (*B*) NFAT transcriptional activity in WT, *mcip1*$-/-$, *mcip2*$-/-$, or 2KO MEFs for the indicated times after thapsigargin (2 μM) treatment. Results were averaged from three independent experiments. *, $P < 0.05$ versus none; #, $P < 0.05$ versus WT MEFs within a treatment period. (*C*) Nuclear occupancy of NFATc1 in WT and 2KO MEFs for the indicated times after thapsigargin treatment (*D*) or Ang II stimulation (100 nM). Results were averaged from three independent experiments. *, $P < 0.05$ versus none; #, $P < 0.05$ versus WT MEFs within a treatment period. (*E*) Western blotting for NFATc1 migration at baseline or after thapsigargin treatment in WT and 2KO MEF extracts. (*F*) Western blotting for CnA, CnB, or GAPDH protein from WT and 2KO MEF extracts. (*G*) Western blotting for CnB after calmodulin (CaM) immunoprecipitation from WT and 2KO MEFs at baseline or after acute thapsigargin stimulation (Ca^{2+}). Control Western blotting for the amount of calmodulin precipitated is also shown.

Loss of MCIP1/2 Attenuates Calcineurin–NFAT Signaling in 2KO Mouse Embryonic Fibroblasts (MEFs). As described, even subtle overexpression of MCIP1 can inhibit calcineurin–NFAT signaling. Here we used an NFAT-dependent luciferase reporter and thapsigargin stimulation to increase intracellular Ca^{2+} in WT MEFs (Fig. 2*A*). However, to evaluate the effect associated with loss of endogenous MCIPs on calcineurin–NFAT signaling, MEFs were generated from *mcip1*$-/-$, *mcip2*$-/-$, and 2KO embryos and compared with identically generated WT MEFs (Fig. 2*B*). The data demonstrate a significant reduction in NFAT transcriptional activity at 4 h in *mcip1*$-/-$, *mcip2*$-/-$, and 2KO MEFs, which was also observed at 2 and 6 h (Fig. 2*B* and data not shown). However, by 8 and 12 h, only *mcip1*$-/-$ and 2KO MEFs showed reduced NFAT transcriptional activity to increased Ca^{2+} compared with WT MEFs (Fig. 2*B*). Similarly, an adenovirus encoding NFATc1-GFP was infected in WT and 2KO MEFs for analysis of acute nuclear translocation after thapsigargin and angiotensin II (Ang II) stimulation. Once again, 2KO MEFs demonstrated reduced calcineurin–NFAT signaling characterized by less efficient nuclear occupancy/translocation in 2KO MEFs versus WT MEFs at 30 min and 2 h (Fig. 2 *C* and *D*). Consistent with these results, Western blotting for the migration rates of total NFATc1 protein showed less dephosphorylation (dephosphorylation increases migration) after thapsigargin stimulation in 2KO compared with WT MEFs (Fig. 2*E*). There was no compensatory alteration in CnA or CnB proteins levels in 2KO MEFs versus WT MEFs (Fig. 2*F*). Assessment of calcineurin activation through its association with calmodulin after thapsigargin stimulation was not altered in 2KO MEFs versus WT MEFs (Fig. 2*G*). Thus, loss of *mcip1/2* in fibroblasts only compromises NFAT activation.

MCIP1/2 Facilitate Calcineurin in the Heart. Deletion of *CnA*β in the mouse severely compromises the ability of the heart to hypertrophy in response to pressure overload and neuroendocrine stimulation (4). More recently, Olson and colleagues (20) deleted the *mcip1* gene in the mouse, which also showed reduced hypertrophy after pressure overload stimulation, suggesting the possibility that endogenous levels of MCIP might have a stimulatory interaction with calcineurin. Here we analyzed mice null for *mcip1* and *mcip2* and 2KOs, as well as mice null for *mcip1/2* $\times$ *CnA*β (3KO). Baseline evaluation of 2KO mice at 2 months or 1 year of age showed no phenotypic manifestations of cardiac disease or dysfunction as assessed by echocardiography and histology (Table 1 and data not shown). Two-month-old *mcip1*$-/-$, *mcip2*$-/-$, and 2KO mice were subjected to pressure overload stimulation by transverse aortic constriction (TAC) for 2 weeks and compared with age- and strain-matched WT mice (Fig. 3*A*). Remarkably, each single null and the 2KO mouse showed significantly less increase in heart weight normalized to tibia length (HW/TL) in response to pressure overload stimulation (Fig. 3*A*). Analysis of myocyte area from histological sections revealed a similar profile of inhibited hypertrophy in the absence of *mcip1/2* (Fig. 3*B*). Analysis of cardiac function by echocardiography over 4 weeks of pressure overload stimulation showed a partial protection from pressure

Table 1. Echocardiography in WT and 2KO mice at 2 months or 1 year

	WT, 2 mo	2KO, 2 mo	WT, 1 year	2KO, 1 year
LVED	3.73 ± 0.29	4.03 ± 0.13	4.00 ± 0.59	4.60 ± 0.40
LVES	2.31 ± 0.24	2.53 ± 0.21	2.60 ± 0.40	3.20 ± 0.50
Septum	1.06 ± 0.12	1.06 ± 0.08	1.00 ± 0.12	1.00 ± 0.08
LV	0.95 ± 0.13	1.02 ± 0.13	1.00 ± 0.13	1.10 ± 0.13
FS, %	50.4 ± 8.8	48.1 ± 5.1	35.7 ± 4.8	29.61 ± 5.1

All measurements are means ± SEM in four mice, and each animal was measured three separate times. Septal and left ventricular (LV) wall thicknesses were assessed in systole and shown as millimeters. LVED, left ventricular end-diastolic dimension; LVES, left ventricular end-systolic dimension; FS, fractional shortening.

overload-induced decompensation in the absence of *mcip1/2* compared with WT mice (Fig. 3*C*), consistent with a similar profile of attenuated decompensation in the absence of *CnAβ*, WT mice treated with cyclosporine A, or MCIP1 overexpressing transgenic mice (5, 24, 25). Interestingly, 2KO mice also did not hypertrophy in response to 2 weeks of phenylephrine plus Ang II infusion by osmotic minipump or after 3 weeks of forced exercise by swimming (Fig. 3 *D* and *E*). Myocardial damage after ischemia-reperfusion injury was also increased in *mcip1*−/− and 2KO mice, similar to our previous observations in *CnAβ*−/− mice (Fig. 3*F*). However, loss of *mcip2* did not increase the infarction area after ischemia-reperfusion injury, suggesting that MCIP1 and MCIP2 function differently in this stress response.

Although the individual or combined loss of *mcip1/2* renders the heart largely resistant to cardiac hypertrophy, a slightly more complex regulatory paradigm was suggested by incorporation of the activated CnA transgene into the *mcip1*−/− and *mcip2*−/− backgrounds. At 3 weeks of age, the activated CnA transgene induced a nearly 2-fold increase in HW/TL in *mcip1/2*+/+ mice, a level of cardiac hypertrophy that was not altered by deletion of *mcip2* (Fig. 3*H*). However, loss of *mcip1* induced a significantly greater increase in cardiac hypertrophy associated with the activated CnA transgene, suggesting that *mcip1* functioned as a repressor in the presence of the activated CnA transgene (Fig. 3*H*). This dichotomous result is likely due to the observation that endogenous levels of MCIP1 are massively augmented by the activated CnA transgene in the heart, rendering MCIP1 inhibitory toward calcineurin similar to MCIP1 overexpression by transgenesis (Fig. 3*G*). In addition, pressure overload and phenylephrine plus Ang II infusion did not detectably increase MCIP1 protein levels in the heart, suggesting that, although calcineurin is activated by these stimuli, it is not of sufficient magnitude to increase MCIP1 protein expression to inhibitory levels (data not shown). Finally, we also crossed both the *mcip1*- and *mcip2*-null alleles into the *CnAβ*-null background. If endogenous MCIP functions as a calcineurin inhibitor, crossing into the *CnAβ*-null background should partially rescue the attenuated profile of hypertrophy after pressure overload associated with the remaining CnA*α* gene. However, the data demonstrate that 3KO have a similar profile of inhibited hypertrophy compared with 2KO and *CnAβ*-null mice (Fig. 3*I*). Collectively, these results further suggest that MCIPs serve to facilitate calcineurin functionality *in vivo*.

MCIP1/2 Facilitate Calcineurin in Skeletal Muscle and T Cells. We have previously shown that loss of *CnAβ* or *CnB* from skeletal muscle severely reduces baseline and exercise-induced slow/oxidative fiber-type switching (10, 26). Given this known function of calcineurin in skeletal muscle, we compared the phenotype of 2KO mice for induction of the slow/oxidative program after 3 weeks of forced swimming. WT mice showed a significant

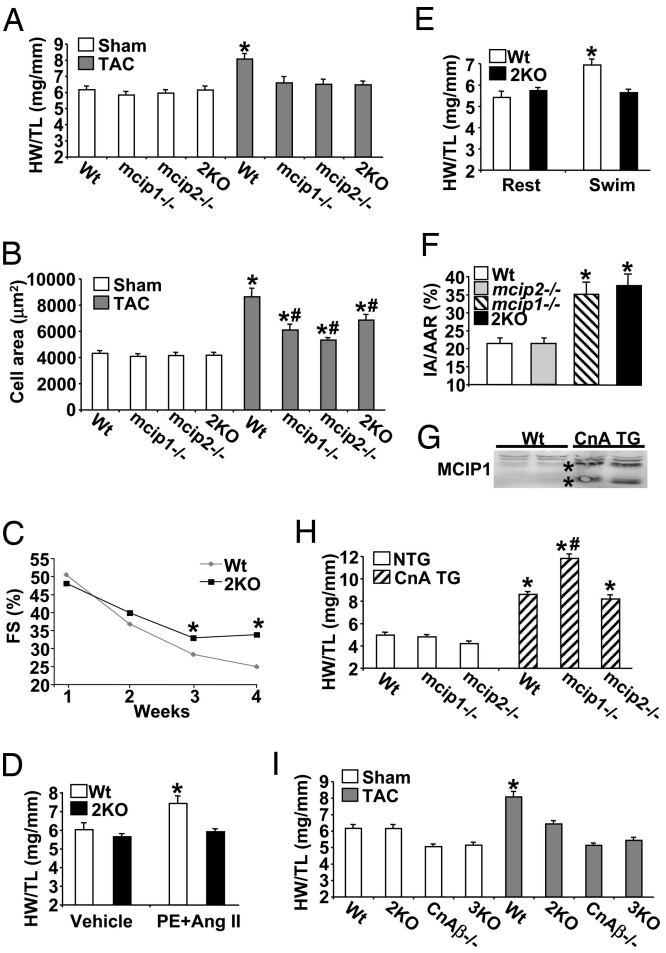

Fig. 3. Assessment of MCIP1/2 function in the heart. (*A*) HW/TL in the indicated groups of mice 2 weeks after a sham or TAC surgical procedure. n = 6–16 mice per group; *, $P < 0.05$ versus WT sham. (*B*) Cardiomyocyte area in the indicated groups of mice 2 weeks after a sham or TAC surgical procedure. n = four hearts analyzed per cohort, with at least 400 cells quantified in total. *, $P < 0.05$ versus WT sham; #, $P < 0.05$ versus WT TAC. (*C*) Echocardiography measured fractional shortening (FS) in WT and 2KO mice at the indicated time points after TAC. At least six mice were analyzed per group at each time point. *, $P < 0.05$ versus WT. (*D*) HW/TL in WT and 2KO mice after 2 weeks of vehicle or phenylephrine plus Ang II infusion. n = 6–12 mice per group; *, $P < 0.05$ versus WT vehicle. (*E*) HW/TL in WT and 2KO mice at rest (n = five to six mice) or after swimming (n = 8–10 mice); *, $P < 0.05$ versus WT rest. (*F*) Infarct area normalized to area at risk (IA/AAR) in WT (n = 6), *mcip1*−/− (n = 7), *mcip2*−/− (n = 6), and 2KO mice (n = 7). *, $P < 0.05$ versus WT. (*G*) Western blotting for MCIP1 protein from the hearts of WT and activated CnA transgenic mice. (*H*) HW/TL in the indicated groups of mice with and without the activated CnA transgene. *, $P < 0.05$ versus WT nontransgenic (NTG); #, $P < 0.05$ versus WT with the activated CnA transgene. (*I*) HW/TL in the indicated groups of mice 2 weeks after a sham or TAC surgical procedure. *, $P < 0.05$ versus WT sham.

increase in type I myosin heavy chain after exercise, a hallmark of the slow/oxidative fiber-type program, yet 2KO mice showed no switching (Fig. 4*A*). Quantitation of these results from six mice per group confirmed lack of fiber-type switching in 2KO mice (Fig. 4*B*). These results are consistent with the hypothesis that MCIPs can facilitate calcineurin function in skeletal muscle.

Loss of *CnAβ* and *CnB* in lymphocytes compromises their activation and/or development (6, 7). To further compare the phenotype of *CnAβ*−/− mice and *mcip1/2*-null mice, lymphocyte development and function were evaluated. FACS of splenocytes from 6-week-old mice demonstrated no reduction in B cells or CD3-, CD4-, or CD8-positive cells in *mcip1*−/−, *mcip2*−/−

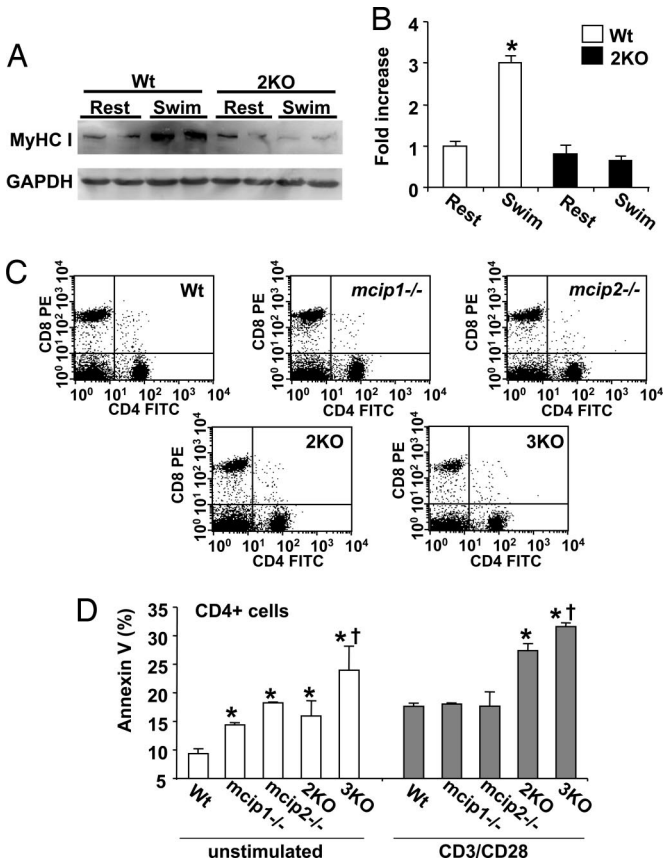

Fig. 4. Assessment of MCIP function in skeletal muscle and T cells. (*A*) Western blotting for type I myosin heavy chain (MyHC I) and GAPDH in WT and 2KO mice at rest or after swimming. (*B*) Quantitation of MyHC I protein levels in the gastrocnemius in three independent experiments, each containing two mice per condition. (*C*) Total splenocytes were isolated and stained with the indicated antibodies and subjected to FACS (Becton Dickinson). FACS was performed on 10,000 cells in each assay, which is representative of six animals per group. (*D*) Assessment of apoptosis levels in purified $CD4^+$ T cells from the indicated mice that were incubated in duplicate 96-well plates with 5 μg/ml αCD3, 2.5 μg/ml αCD28 antibodies for 60 h at 37°C. Results are shown as percentage of gated live cells. *, $P < 0.05$ versus WT; †, $P < 0.05$ versus 2KO.

or 2KO mice (Fig. 4*C* and Table 2). These results are also consistent with previous observations made in *mcip1*−/− mice (21). However, consistent with our previous observations in *CnAβ*−/− mice, T cell development was compromised in the 3KO mice (Fig. 3*C* and Table 2). To evaluate T cell activation, $CD4^+$ T cells were purified and stimulated with CD3/CD28 antibody for assessment of proliferation rates and cytokine production. Loss of *mcip1*−/−, *mcip2*−/−, or both genes together did not reduce $CD4^+$ T cell activation, IL-4, or IFN-γ production (data not shown). However, phorbol 12-myristate 13-acetate/ionomycin stimulation of total splenocytes from 2KO, *CnAβ*−/−, and 3KO mice showed reduced proliferation and IL-2 production compared with WT (data not shown). Loss of *mcip1* was previously shown to increase $CD4^+$ T cell apoptosis (21). Similarly, here we identified increased $CD4^+$ T cell annexin V labeling at baseline or after CD3/CD28 stimulation in *mcip1*−/−, *mcip2*−/−, 2KO, and 3KO mice (Fig. 4*D*). Importantly, loss of *CnAβ* did not rescue the increase in annexin V labeling associated with *mcip1/2* deletion but instead significantly increased it (Fig. 4*D*). This latter result further supports the hypothesis that MCIPs are permissive for calcineurin signaling *in vivo* (see *Discussion*).

MCIP1/2 Facilitate Calcineurin in the Brain. Targeted deletion of *CnB1* in the brain resulted in a phenotype of hyperactivity and working memory (short-term memory) deficits in mice (11, 12). However, overexpression of activated calcineurin in the brain led to a distinct phenotype whereby only long-term memory was affected (27). To further associate the role of MCIPs in the brain with the known effects of calcineurin inhibition, behavioral and activity analyses were performed in 2KO mice. Consistent with *CnB1*-deleted mice, 2KO mice showed a significant increase in cumulative activity during light and dark conditions (Fig. 5*A*). *CnAβ*−/− mice showed an even greater increase in total locomotor activity, although this effect was not reduced in 3KO mice (Fig. 5*A*). Total contacts with an exercise wheel were also dramatically increased in 2KO mice compared with WT mice (Fig. 5*B*). Also of interest, 2KO mice showed faster overall movements in an open field test compared with WT mice (Fig. 5 *C* and *D*). As a control, 2KO mice did not show an alteration in their percentage of crossings in an open field test or time spent in the corners, suggesting that anxiety/stress levels were not altered in 2KO versus WT mice (Fig. 5*E* and data not shown). Finally, 2KO mice showed a subtle but significant impairment in working memory compared with WT mice, as assessed by novel and familiar object recognition analysis at 24 h (Fig. 5*F*). However, 2KO mice did not show a defect in long-term memory (data not shown). Thus, loss of *mcip1/2* in the brain also appears to render a phenotype that resembles reduced calcineurin activation, further suggesting that MCIP proteins facilitate calcineurin signaling *in vivo*.

Discussion

Here we presented multiple lines of evidence suggesting that the physiologic function of MCIP is to facilitate, or at least permit, calcineurin signaling *in vivo*. In heart, brain, skeletal muscle, and T cells, loss of *mcip1/2* in mice led to a phenotype similar to mice lacking *CnAβ*, whereas the combined deletion of *mcip1/2* with *CnAβ* did not suppress the 2KO phenotype in any of the tissues examined. MEFs null for *mcip1, mcip2*, or 2KO demonstrated a clear profile of reduced calcineurin–NFAT coupling. One unique caveat to these observations is that *mcip2* deletion reduced calcineurin-NFAT signaling only at early time points after thapsigargin stimulation, whereas at later time points, MCIP1 appeared to be more important, suggesting some functional differences between MCIP1 and MCIP2. It is also impor-

Table 2. Quantitation of lymphocyte numbers from the spleens of the indicated genotypes

	WT	*mcip1*−/−	*mcip2*−/−	2KO	3KO
CD19/CD220	45.2 ± 3.9	47.3 ± 2	51.7 ± 2.8	53.3 ± 2.29	59.4 ± 2.22
CD3	39.3 ± 4.4	40 ± 3.17	36.6 ± 2.63	35.4 ± 2.27	25.1 ± 2.1
CD4	18.4 ± 1.5	22.48 ± 1.8	22.7 ± 0.97	19.8 ± 1.3	14.5 ± 1.1
CD8a	19.6 ± 2.3	16.4 ± 1.5	12.9 ± 1.18	14.2 ± 1.42	7.4 ± 0.6

Total splenocytes were isolated from 6-week-old mice of the indicated genotype (five to six each) and subjected to FACS to quantify the percent of cells with reactivity against the indicated antibodies. T cell numbers were reduced only in the absence of *CnAβ*. Results are expressed as percent of gated cells ± SEM.

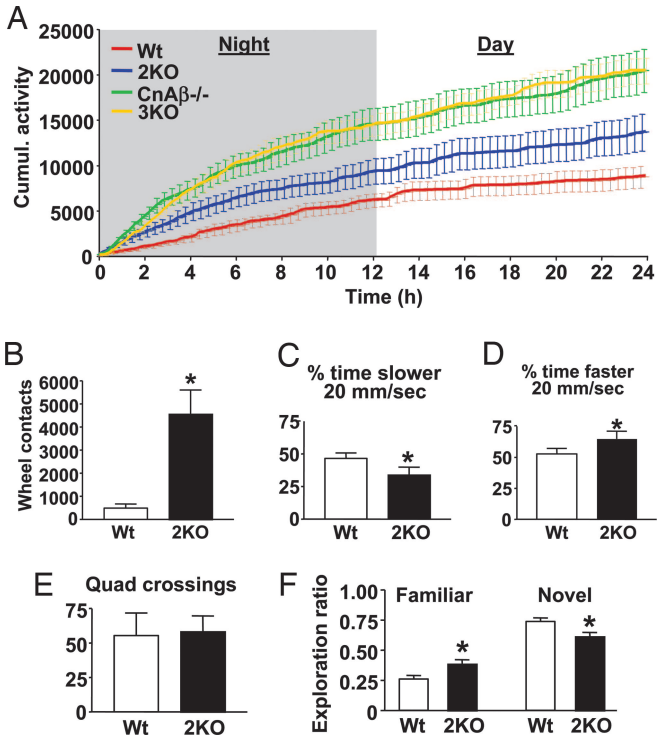

Fig. 5. Assessment of MCIP function in the brain. (*A*) Total cumulative motor activity during the dark and light phase over 24 h in the indicated genotypes of cage-housed mice (n = six mice per group). (*B*) Total number of running wheel contacts over 3–4 days in WT and 2KO mice housed in cages (n = six mice per group; *, $P < 0.05$ versus WT). (*C* and *D*) Percentage of time spent moving faster or slower than 20 mm per sec in an open-field test in WT and 2KO mice (n = six mice per group; *, $P < 0.05$ versus WT). (*E*) Percentage of movements that involves complete crossing of an open field in WT and 2KO mice (n = six mice per group). (*F*) Time exploration ratio of a familiar and novel object with WT and 2KO mice after 24 h (n = six mice per group; *, $P < 0.05$ versus WT).

tant to note that loss of *mcip1/2* did not alter calcineurin activity *per se* or its association with calmodulin, and in fact, overexpression of MCIP1 always served an inhibitory function *in vivo* and *in vitro*. Hence, it remains uncertain exactly how endogenous MCIPs facilitate calcineurin activity toward target proteins, although inhibitor-2 and DARPP-32 may play a similar role in permitting PP1 activity through an unknown mechanism (28, 29).

Many previous reports implicating MCIP as a calcineurin inhibitor have relied on overexpression approaches (17, 21–23, 25, 30–34). However, there is ample precedent in the literature that overexpression of a kinase or phosphatase docking/activating protein can actually have an inhibitory function based on a sequestration effect. For example, overexpression of the c-Jun N-terminal kinase (JNK)-interacting protein (JIP1) results in JNK inhibition, although endogenous levels of JIP1 normally function in specifically localizing and enhancing JNK activity within a cell (35). A kinase anchoring protein 79 (AKAP79), which docks protein kinase A, protein kinase C, and calcineurin to facilitate their selective and coordinated activity for localized substrates, can also function as a repressor when overexpressed (36, 37). Even more germane endogenous levels of inhibitor-2 protein (yeast homologue Glc8) were shown to function as facilitators of protein phosphatase 1 (PP1), although overexpression of inhibitor-2 blocks PP1 activity (28, 38). Phosphorylation of inhibitor-2 can alter its inhibitory characteristics toward PP1, not unlike the ability of specific kinases to alter MCIP1 function toward calcineurin by phosphorylation (19, 39). All these observations are particularly interesting given the relative conservation between the calcineurin docking domains in MCIP1 and NFAT proteins versus the docking domain in PP1 for inhibitor-2 and other interacting proteins (40). Deletion of *rcn1* in yeast (MCIP homologue) produced a phenotype that was similar to Rcn1 overexpression, suggesting that both states inhibited calcineurin and that the true physiologic function of Rcn1 *in vivo* may be to facilitate calcineurin activity toward substrates (17). In support of this hypothesis, *C. neoformans* mutant for *cbp1* (MCIP homologue) displays a phenotype that is similar to, albeit less severe than, calcineurin mutants (18). Finally, two separate reports have examined the effect of *mcip1* deletion in the mouse. Olson and colleagues (20) observed depressed cardiac hypertrophy in *mcip1*-null mice after pressure overload stimulation or isoproterenol infusion, consistent with the hypothesis that MCIP1 is required to facilitate calcineurin signaling. McKeon and colleagues (21) reported that $CD4^+$ T cells from *mcip1*-null mice had an increase in the ratio of dephosphorylated to phosphorylated NFATc2 and reduced IL-2 production after T cell receptor stimulation at specific dosages of CD3 antibody, suggesting that MCIP1 might serve an inhibitory role under certain conditions in these cells. In support of this result, partial reduction of MCIP1 protein using an antisense RNA approach increased NFAT activity and inflammatory response gene expression in endothelial cells (41).

Although the functional role that we have proposed for MCIPs would appear to contradict results from McKeon and colleagues (21), there are many points of similarity in the data. Similar to McKeon and colleagues, we also observed an increase in apoptosis rates in *mcip1/2*-null $CD4^+$ T cells after CD3 stimulation and no obvious defects in T cell development. However, the most significant difference between our studies is related only to interpretation of data, because loss of *CnAβ* in the *mcip1/2*-null background did not rescue the phenotype, consistent with the proposal that MCIPs permit/facilitate calcineurin activity. By comparison, McKeon and colleagues (21) observed that cyclosporine A rescued the increase in cell death associated with *mcip1* deletion, although cyclosporine A can also block cell death through a calcineurin-independent function involving inhibition of mitochondrial permeability transition.

Although *CnAβ−/−* mice had a phenotype similar to 2KO mice in the heart, skeletal muscle, brain, and T cells, there were some minor differences at baseline. 2KO mice showed no differences in CD4 or CD8 T cell numbers in the spleen or thymus (data not shown), yet *CnAβ−/−* mice were previously shown to have a substantial reduction in both T cell populations at baseline (7). These results indicate that loss of *mcip1/2* does not impact T cell development the same as loss of *CnAβ*, possibly suggesting that MCIPs regulate at different times in T cell maturation or that MCIP facilitates only a select range of calcineurin targets. Similarly, loss of *mcip1/2* did not affect basal type I myosin heavy chain protein expression in skeletal muscle (only exercise-induced expression), whereas *CnAβ−/−* and *CnB1*-null mice each showed dramatic reductions in basal expression (10, 26). With respect to the brain, loss of *CnAβ−/−* produced a more pronounced increase in cumulative locomotor activity compared with a significant, albeit more modest, effect in 2KO mice. This observation suggests that loss of *mcip1/2* does not fully compromise calcineurin activation under all conditions or toward all targets. A similar conclusion was proposed based on the observations in *C. neoformans* whereby *calcineurin* mutants had a more severe phenotype than did *cbp1* mutants (18). The results observed in mammals also need to be qualified by two additional considerations. Mammals contain a third *mcip* gene (*mcip3*) that is also expressed in brain and lymphocytes and could contribute to residual calcineurin target facilitation in the absence of *mcip1/2* (42). MCIPs have also been reported to interact with proteins other than calcineurin, suggesting the

possibility of calcineurin-independent effects. For example, MCIP1 was shown to immunoprecipitate with integrins and Raf-1 (43, 44). Thus, MCIPs are likely to function as calcineurin facilitators through direct and indirect mechanisms.

Methods

Generation of *mcip1*−/− and *mcip2*−/− Mice. A more extensive description of the targeting strategy and strain background, as well as many other procedures, is given in *Supporting Text*, which is published as supporting information on the PNAS web site.

Western Blotting and Immunoprecipitation. Generation of protein samples from tissue and cultured cells, along with Western blotting and chemifluorescent detection, was performed as described (4, 7, 45). Calmodulin immunoprecipitation for calcineurin binding in MEF protein extracts (500 μg) was performed as described (46).

Cell Culture. MEFs were isolated at embryonic day 12.5 from 2KO or WT matings, as described (47). Cultures were harvested 24 h after recombinant adenoviral infection, and luciferase assays were performed as described (48).

Cardiac Hypertrophy Measurements *in Vivo*. See *Supporting Text*.

Murine TAC, Agonist Infusion, Exercise, and Ischemia/Reperfusion Protocols. Performed as described (48, 49) and see *Supporting Text*.

Preparation of Lymphocytes. See *Supporting Text*.

Activity and Memory Assessment. Home cage activity was measured via TSE (TSE Systems, Midland, MI) dual array of infrared photo beams, using TSE software to assess gross movement and rearing behaviors, as described (50). Additional tests are described in *Supporting Text*.

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