Published in final edited form as:
Science. 2008 January 25; 319(5862): 476–481. doi:10.1126/science.1151227.

NFAT Binding and Regulation of T Cell Activation by the Cytoplasmic Scaffolding Homer Proteins

Guo N. Huang[1,2,*], **David L. Huso**[3,†], **Samuel Bouyain**[4,†], **Jianchen Tu**[2,†], **Kelly A. McCorkell**[5,†], **Michael J. May**[5], **Yuwen Zhu**[6], **Michael Lutz**[7], **Samuel Collins**[7], **Marlin Dehoff**[2], **Shin Kang**[2], **Katharine Whartenby**[7], **Jonathan Powell**[7], **Daniel Leahy**[4], and **Paul F. Worley**[2,8,‡]

[1]Program in Biochemistry, Cellular and Molecular Biology, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA.

[2]Solomon H. Snyder Department of Neuroscience, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA.

[3]Department of Molecular and Comparative Pathobiology, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA.

[4]Department of Biophysics and Biophysical Chemistry, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA.

[5]Department of Animal Biology, University of Pennsylvania, Philadelphia, PA 19104, USA.

[6]Department of Dermatology, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA.

[7]Department of Oncology-Immunology/Hematopoiesis, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA.

[8]Department of Neurology, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA.

Abstract

T cell receptor (TCR) and costimulatory receptor (CD28) signals cooperate in activating T cells, although understanding of how these pathways are themselves regulated is incomplete. We found that Homer2 and Homer3, members of the Homer family of cytoplasmic scaffolding proteins, are negative regulators of T cell activation. This is achieved through binding of nuclear factor of activated T cells (NFAT) and by competing with calcineurin. Homer-NFAT binding was also antagonized by active serine-threonine kinase AKT, thereby enhancing TCR signaling via calcineurin-dependent dephosphorylation of NFAT. This corresponded with changes in cytokine expression and an increase in effector-memory T cell populations in Homer-deficient mice, which also developed autoimmune-like pathology. These results demonstrate a further means by which costimulatory signals are regulated to control self-reactivity.

[‡]To whom correspondence should be addressed. pworley@jhmi.edu.
[*]Present address: Department of Molecular Biology, University of Texas Southwestern Medical Center, Dallas, TX 75235, USA.
[†]These authors contributed equally to this work.

Supporting Online Material
www.sciencemag.org/cgi/content/full/319/5862/476/DC1
Materials and Methods
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Table S1

T cells are activated through the TCR and costimulatory pathways predominantly mediated by the cell surface receptor CD28. Although these pathways are relatively well defined, questions still remain about how costimulatory signals are regulated. The Homer family of cytoplasmic scaffolding proteins are known to function at the neuronal excitatory synapse (1, 2), although their wide tissue distribution, including within the immune system, suggests that their functions may be relatively broad.

To investigate the in vivo functions of the Homer proteins, we generated mice in which the loci for each Homer gene were deleted (Homer1, 2, and 3). Of these, we noted that the Homer3-deficient mice (3) displayed lymphocyte infiltration of multiple organs and hyperplasia in lymph nodes by 10 weeks of age (fig. S1), which suggested that at least one of the family might possess some level of immune function. Because Homer proteins typically have redundant roles (1, 2), we first assessed their possible role in T cell activation, by assaying interleukin-2 (IL-2) production in T cells lacking all three genes (TKO). IL-2 production was increased by a factor of 2 to 6 in anti-CD3–stimulated T cells from Homer TKO mice relative to wild-type controls (Fig. 1A). By contrast, when T cells were activated by costimulation of both CD3 and CD28, no measurable difference in IL-2 production was detected between wild-type and Homer-deficient mice (fig. S2).

To examine the potential role of Homer proteins in T cell activation in more detail, we used short hairpin RNAs (shRNAs) to knock down Homer gene expression in human Jurkat T cells (Fig. 1B). Knockdown of Homer2 or Homer3, but not Homer1, enhanced the expression of a luciferase reporter driven by the IL-2 promoter by a factor of 3 to 6 (Fig. 1C). Homer2 and Homer3 appeared to have redundant functions in these assays because overexpression of Homer2, but not Homer1, could rescue the loss of Homer3 (Fig. 1D). The IL-2 promoter integrates signals from the calcineurin-NFAT, MAPK-AP1, and NF-κB pathways (4, 5); to identify which pathways might be regulated by Homer, we used luciferase reporter constructs under the control of multimerized binding elements for individual transcription factors. The calcineurin-NFAT pathway was preferentially enhanced in cells depleted of Homer2 or Homer3 (Fig. 1E). To respond to calcium signals, NFAT is first dephosphorylated by calcineurin (4), and in Jurkat T cells that expressed shRNAs targeting Homer3, enhanced dephosphorylation of the NFATc2 isoform was observed after activation but not under basal conditions (Fig. 1F). No difference was detected in key signaling events for other pathways such as Fos and Jun induction for the AP1 pathway or IκB degradation for the NF-κB pathways (Fig. 1F); these results supported the notion that Homer2 and Homer3 function to selectively inhibit calcineurin-NFAT activation.

The upstream events of NFAT dephosphorylation include calcium mobilization and activation of calcineurin. In a comparison of the calcium dynamics in response to CD3 activation in wild-type and Homer TKO primary T cells, no difference was detected (fig. S3A).An analysis of calcineurin activity in Homer3-depleted Jurkat T cells showed normal calcium-dependent phosphatase activity (fig. S3B). These results suggested that the Homer proteins function downstream of calcineurin activation but upstream of NFAT dephosphorylation.

We next asked how Homer might inhibit NFAT activation. Coexpression of a green fluorescent protein (GFP)–labeled form of NFATc2 (GFP-NFATc2) with Homer2 or Homer3, but not Homer1, resulted in large cytoplasmic punctae containing both NFATc2 and Homer (Fig. 2A and fig. S4), suggesting physical interaction of the proteins. In support of the notion that Homer directly binds NFAT, purified GST-Homer2 and GST-Homer3 fusion proteins could pull down NFATc1, c2, and c4 isoforms expressed in human embryonic kidney (HEK) cells (Fig. 2B). Homer1 bound only NFATc4, whereas NFATc3

did not show binding to any of the Homer proteins (Fig. 2B). In addition, Homer3 and NFATc2 could be coimmunoprecipitated from whole T cell lysates (Fig. 2C).

We next compared Homer and calcineurin binding to NFATc2. When coexpressed, similar amounts of Homer3 and calcineurin bound GST-NFATc2 (Fig. 2D), which suggested that they have comparable affinities. GST-Homer3 bound NFAT robustly even in calcium-depleted buffers (fig. S5A) and was not dependent on NFAT phosphorylation (fig. S5B). NFATc2 contains a DNA binding domain (DBD) and an N-terminal regulatory domain that includes two calcineurin docking sites, serine-arginine–rich regions (SRR), serine-proline–rich regions (SP), and a nuclear localization sequence (NLS) (Fig. 2E) (6). Binding assays indicated that Homer3 binds a broad region in the N-terminal regulatory domain (Fig. 2E). Because the Homer binding region overlaps with the calcineurin docking sites on NFAT, we examined whether any of the Homer proteins might compete with calcineurin for NFATc2 binding. Using purified recombinant proteins, we found that Homer3 reduces calcineurin binding to NFATc2 (Fig. 2F).

For all previously described Homer interactions, Homer1, 2, and 3 proteins show identical properties (1, 2). Accordingly, we examined the structural basis of Homer binding to NFATc2 that distinguishes Homer2/3 from Homer1. Binding required the EVH1 domain of Homer3 (Fig. 3, A and B), and mutation analysis identified five amino acids that are most critical (Fig. 3C). The crystal structure of Homer3 was solved and revealed that all five amino acids cluster at one prominence that is remote from the surface used by Homer to bind polyproline-rich sequences (Fig. 3D) (7). We noted a threonine and two serines (Thr^{36}, Ser^{38}, and Ser^{52}) close to the putative NFATc2 binding surface (fig. S6) that are predicted AKT phosphorylation targets (3). Indeed, activated AKT (8, 9) prevented the formation of cytoplasmic punctae in cells between Homer2 or 3 and GFP-NFATc2 (fig. S7) and reduced Homer2 and 3 binding to GST-NFATc2 in vitro (Fig. 3, E and F). Mimicking phosphorylation of one or more of Thr^{36}, Ser^{38}, and Ser^{52} reduced NFATc2 binding (fig. S8), whereas the triple alanine mutant (in which Thr^{36}, Ser^{38}, and Ser^{52} are all mutated to Ala) bound NFATc2 and the inhibition by AKT was attenuated (fig. S8). AKT is activated by the CD28 pathway (10). CD28 stimulation reduced NFATc2-Homer coimmunoprecipitation from T cells and was AKT-dependent (Fig. 3G). By contrast, treatment with LiCl, a GSK3 inhibitor, did not change the amount of NFATc2 that coimmunoprecipitated with Homer3. These results identify a novel binding surface for Homer and support a model in which CD28/AKT activation reduces Homer3 binding to NFATc2.

To further explore the role of Homer in cooperative activation by CD3 and CD28, we monitored IL-2 expression by T cells isolated from Homer2,3 double-knockout (DKO) mice (3) (Fig. 4A). At a threshold concentration for CD3 activating antibody (1 μg/ml), addition of low concentrations of CD28 antibody resulted in IL-2 expression from Homer2,3 DKO greater than from wild-type T cells by a factor of as much as 9 (Fig. 4A). Homer2,3 DKO T cells also showed enhanced proliferation in response to CD3, either alone or with low-dose CD28 antibody (fig. S9, A and B). By contrast, at a high concentration of CD28 antibody, which is not alone sufficient to induce IL-2 production, addition of CD3 antibody resulted in dose-dependent increases of IL-2 production that were identical in wild-type and Homer2,3 DKO T cells (Fig. 4A). Homer2,3 DKO T cells also showed enhanced (versus wild-type) induction of other NFAT-responsive genes to CD3 activation, including IL-4 and interferon-γ (Fig. 4B). Egr-1, which is not NFAT-responsive (11), was not differentially induced. IL-2 production and proliferation after T cell activation by antigen-presenting cells (APCs) were similar in wild-type$^{6.5}$ and Homer DKO$^{6.5}$ T cells that express TCRs specific for the influenza hemagglutinin antigen (HA) (Fig. 4C and fig. S9C);however, when CD28 signaling was reduced by pretreatment with CTLA4– immunoglobulin (Ig), Homer2,3 DKO

T cells showed a factor of 4 or greater increase in IL-2 production (Fig. 4D). To assay for in vivo effects of APC-dependent T cell activation, we injected cells from Homer2,3 DKO$^{6.5}$ and control transgenic mice into host mice that express the HA antigen on pulmonary epithelial cells (12). Mice died more rapidly when they received Homer2,3 DKO$^{6.5}$ T cells than when they received control T cells (Fig. 4E), consistent with enhanced activation of Homer2,3 DKO T cells.

Thymocyte development appeared normal in Homer2,3 DKO mice (fig. S10); however, the percentage of CD62L^{-}CD44^{high} effector-memory T cells in 7- to 10-week-old mice increased by a factor of 2 to 3.5 relative to age-matched wild-type controls (Fig. 4F), suggesting an increase of autoreactive T cells. T_{reg} cells, which suppress T cell activation and are also dependent on calcineurin-NFAT (13), were slightly increased in Homer2,3 DKO mice (fig. S11). To assess the long-term effects of Homer2,3 DKO, we examined 14-month-old mice for evidence of immune dysfunction. Homer2,3 DKO mice differed from wild-type mice in having more severe mononuclear infiltrative lesions in the liver (5/5 mild-moderate versus 1/5 mild) (Fig. 4G) and in the lacrimal (Harderian) gland (3/5 moderate-severe versus 0/5) (Fig. 4H), which showed evidence of tissue injury. These changes are similar but less severe than those observed in the Homer3 KO mice in mixed B6/129 background (fig. S1), which suggests that genetic background can affect the phenotype.

Our results support a model in which Homer2 and Homer3 are negative regulators of NFAT-dependent signaling in T cells. NFAT also plays a critical role in calcium-dependent signaling in other cell types, including muscle (4) and neurons (14, 15). Interestingly, a *Drosophila* Homer2 homolog was recently identified in a genome-wide RNA interference screen of S2 cells as a negative regulator of NFAT activation (16). Our model anticipates that in the resting state, Homer binds NFATc1/c2 together with the Ca^{2+} release apparatus at the immune synapse (fig. S12). In the condition of CD3 monostimulation of T cells, active calcineurin must compete with Homer2,3 for access to NFAT so that only a fraction of NFAT is dephosphorylated and activated. Because calcineurin activity is required to dephosphorylate NFAT, even in the absence of Homer2,3, calcineurin-NFAT signaling is not altered in the basal state (figs. S13 and S14). When CD28 is coengaged, Homer2,3 dissociates from NFAT, leaving greater access for calcineurin to dephosphorylate NFAT1c/2c. We propose that AKT-dependent reversal of Homer's inhibitory function is one means by which CD28 activation enhances IL-2 production. Homer2 is rapidly up-regulated after T cell activation (17) (fig. S15), which suggests that the balance of CD3 and CD28 required for activation may be dynamically regulated. Although our analysis of Homer deficient mice revealed a relatively modest phenotype, further exploration using other models may reveal more about Homer proteins in regulating T cell responses. In particular, the role of Homer2,3 in establishment of anergy and responses to natural antigens will be important to examine.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank J. Liu for plasmid NFATc2-GFP, plasmid HA-mNFATc2(1–460)–GFP, NFAT-luc, AP1-luc, NF-κB–luc, pSV40–β-galactosidase, and bacteria-expressed calcineurin A and B subunits; A. Rao for plasmid HA-NFATc2 wild-type and CA; D. Ginty for Myr-Akt construct; G. Crabtree for plasmids Flag-NFATc1, Flag-NFATc2, Flag-NFATc3, and GFP-NFATc4; and D. Pardoll for the 6.5 TCR transgenic mice. Supported by National Institute on Drug Abuse grants DA00266 and DA10309 (P.F.W.). The coordinates of the Homer3 EVH structure have been deposited in the Protein Data Bank under accession code 2P8V.

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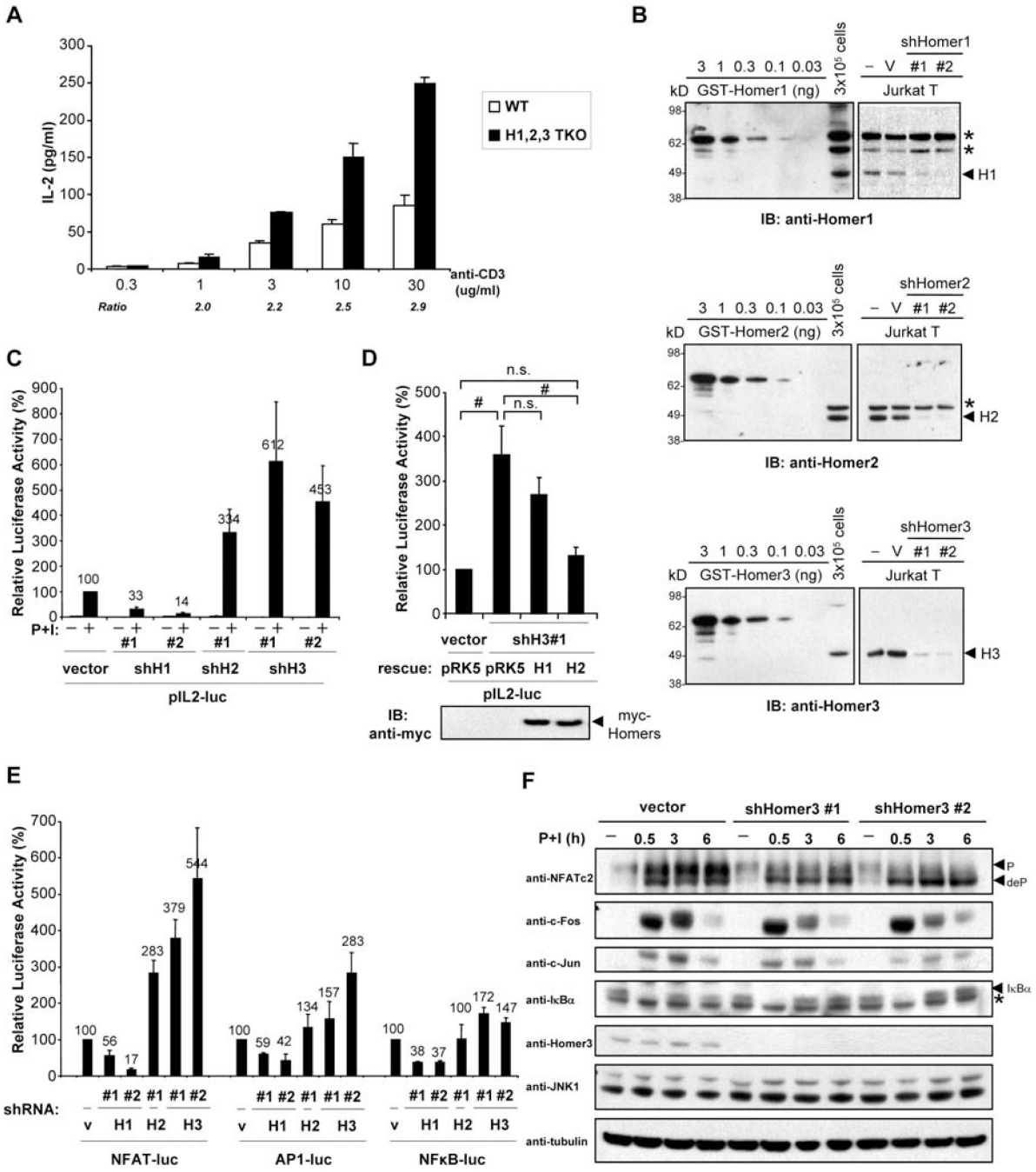

Fig. 1. Homer2 and Homer3 negatively regulate IL-2 expression by inhibiting calcineurin-NFAT pathway activation

(**A**) IL-2 production is enhanced from purified primary Homer1,2,3 triple-knockout (H1,2,3 TKO) T cells after stimulation with anti-CD3 for 24 hours. This result is representative of at least three independent experiments with IL-2 production increases by factors of 4 to 6 from knockout T cells. (**B**) All three Homer family members express in human Jurkat T cells and can be efficiently knocked down by shRNA. (**C**) Depletion of Homer2 or Homer3 enhances IL-2 promoter activity. Jurkat cells were transfected with a luciferase reporter under the control of the IL-2 promoter and Homer shRNA-expressing plasmids and stimulated with PMA (50 ng/ml) and 1 μM ionomycin (P+I). Error bars are SDs from three to seven

independent experiments. (**D**) Homer2 and Homer3 have redundant functions in IL-2 expression regulation. Error bars are SDs from three independent experiments; # denotes $P < 0.05$, and n.s. indicates $P > 0.05$ (Student *t* test). (**E**) Knockdown of Homer2 or Homer3 mainly increases calcineurin-NFAT pathway activation. Cells were stimulated with P+I. Error bars are SDs from three to eight independent experiments. (**F**) Depletion of Homer3 enhances NFAT dephosphorylation. This result is representative of at least three independent experiments. The asterisks in (B) and (F) label the nonspecific cross-reactive proteins.

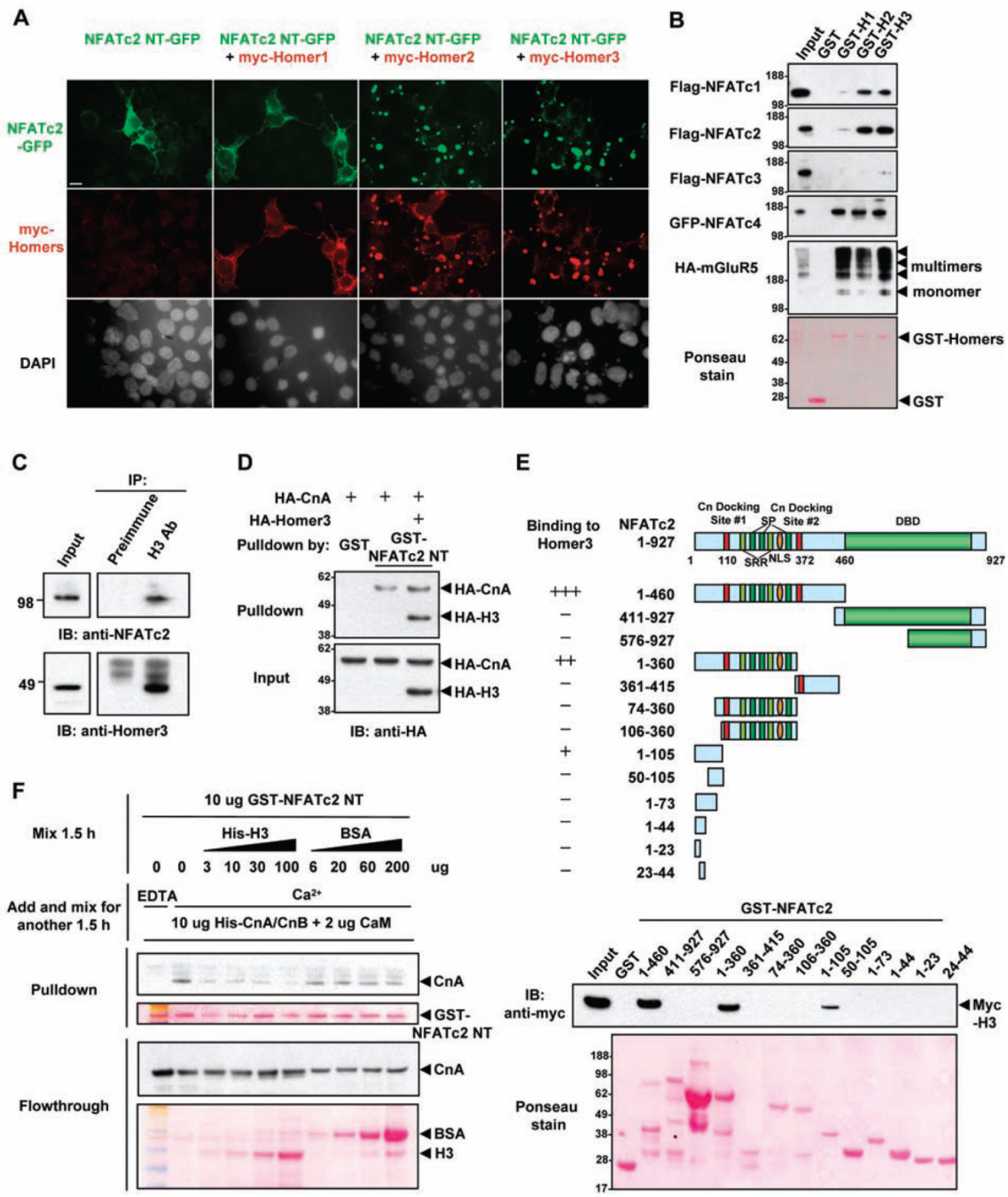

Fig. 2. Homer2 and Homer3 bind NFATc2 and compete with calcineurin for NFATc2 binding

(**A**) NFATc2 forms cytoplasmic punctae in HEK293 cells in the presence of Homer2 or Homer3 overexpression. NFATc2 (1–460) (designated as NT)–GFP is shown in green; Homer proteins are stained in red by an anti-myc mouse antibody. (**B**) Binding specificity between Homer and NFAT family members. mGluR5 binds equally well to Homer1, 2, and 3. (**C**) Coimmunoprecipitation of endogenous Homer3 and NFATc2 from primary mouse T cells. (**D**) GST-NFATc2 NT pulls down comparable amounts of Homer3 and calcineurin. Calcium-containing buffers were used for pull-down assays, and both Homer3 and

calcineurin were detected by an antibody to HA. (**E**) Mapping of regions in NFATc2 that bind Homer3. (**F**) Competition between Homer3 and calcineurin for NFATc2 binding.

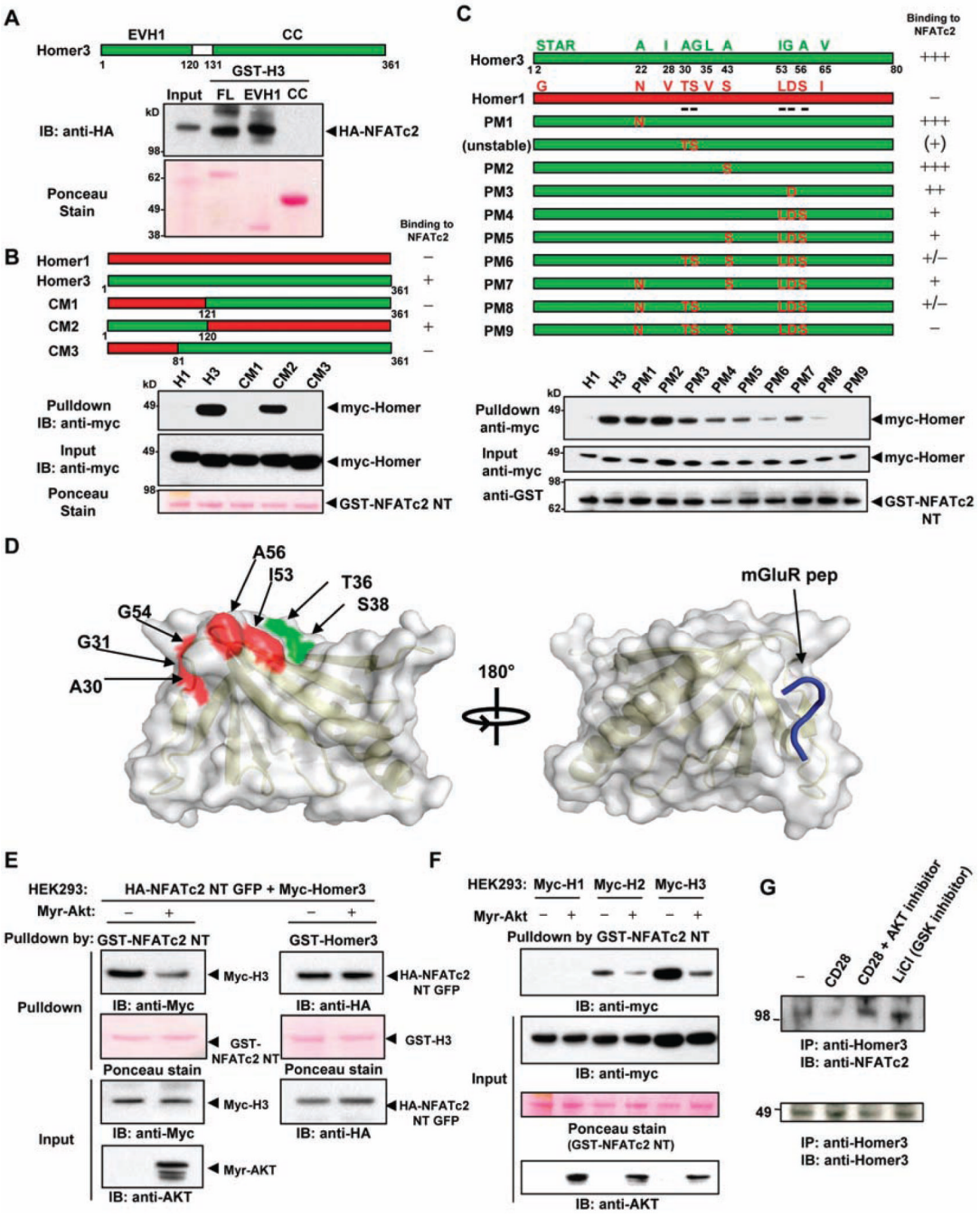

Fig. 3. Five amino acids in the Homer EVH domain distinguish Homer3 from Homer1 for NFATc2 binding

(**A**) The Homer3 EVH domain is sufficient and necessary to bind NFATc2. (**B**) Analysis of Homer3 Homer1 chimeric mutants (CM) reveals that the N-terminal 80 amino acids are critical for NFATc2 binding. (**C**) Analysis of point mutants (PM) of Homer3 with amino acids converted to those in Homer1 for NFATc2 binding. The numbers below the schematic protein bars in (B) and (C) correspond to the positions of the amino acids in Homer3. The five amino acids that are essential for NFATc2 binding are marked by short black bars. (**D**) Crystal structure of the Homer3 EVH1 domain. The five critical amino acids for NFAT binding are labeled in red, and the surface that binds the proline-rich sequence such as

mGluR peptide (pep, in blue) is shown. (**E**) Overexpression of active AKT attenuates Homer3-NFATc2 binding. (**F**) Effect of active AKT on NFATc2 binding to Homer family members. (**G**) Activation of CD28 signaling reduces Homer3-NFATc2 binding in primary mouse T cells. T cells were stimulated for 3 hours by anti-CD28 precoated on dishes at 10 μg/ml. An AKT inhibitor (20 μM) or a GSK inhibitor (10 mM LiCl) was added in the medium as indicated.

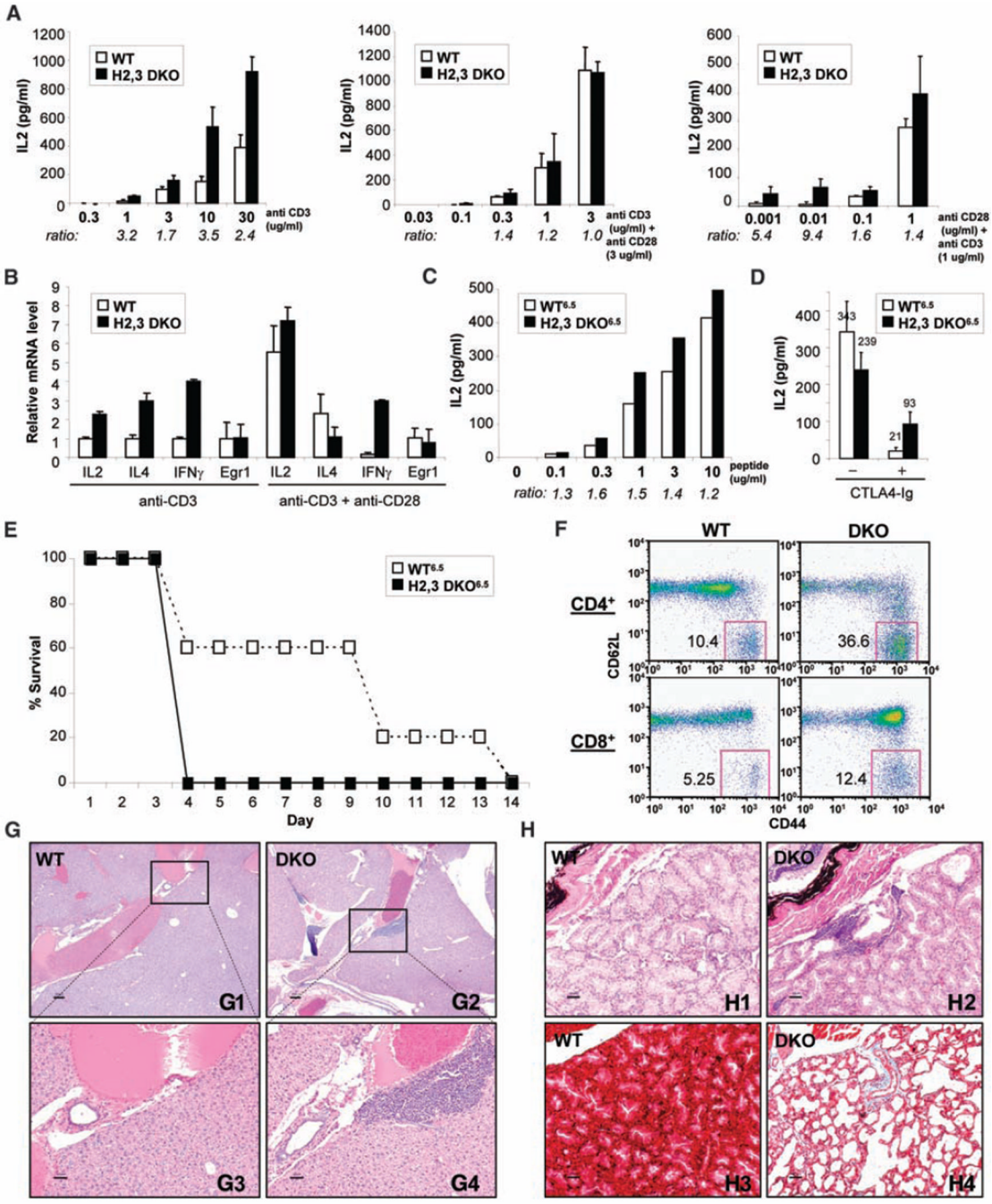

Fig. 4. Deletion of Homer2,3 is linked to autoimmune phenotypes

(**A** and **B**) The costimulatory pathway activation attenuates the difference of IL-2 production between wild type (WT) and Homer2,3 DKO T cells. (A) IL-2 production from purified spleen T cells from wild-type and DKO mice upon stimulation with various dosages of anti-CD3 or anti-CD3 plus anti-CD28. (B) Real-time polymerase chain reaction analysis of gene expression of purified primary T cells stimulated by anti-CD3 (10 μg/ml) or anti-CD3 (3 μg/ml) plus anti-CD28 (3 μg/ml) for 24 hours. (**C** and **D**) IL-2 production from purified $CD4^+$ T cells from wild-type transgenic ($WT^{6.5}$) and Homer2,3 DKO transgenic mice (Homer2,3 $DKO^{6.5}$) 48 hours after mixing with irradiated wild-type APCs in the presence of different

concentrations of HA peptide (C) or HA peptide (10 μg/ml) plus CTLA4-Ig protein (5 μg/ml), which blocks the CD28 signaling (D). (**E**) Homer2,3 DKO$^{6.5}$ T cells promote an accelerated response in a pneumonitis autoimmune model. The data of all mice that did not survive are presented. $P = 0.037$ in a log-rank one-way test (WT$^{6.5}$, n =5;Homer2,3DKO$^{6.5}$, $n = 4$). (**F**) Flow cytometry data showing the staining of the effector-memory T cells (CD62L^{-}CD44^{high}) from 7- to 10-week-old wild-type or DKO splenocytes. The data are representative of three independent experiments in which a total of six animals of each genotype were analyzed. (**G** and **H**) Hematoxylin and eosin stain of the liver (G) and lacrimal gland (H1, H2) sections from 14-month-old wild-type and Homer2,3 mutant mice; trichrome stain of the lacrimal gland sections from a wild-type (H3) and a mutant mouse exhibiting atrophic appearance (H4). Collagen deposition is labeled in blue. Scale bars, 200 μm in (G1) and (G2); 50 μm in (G3), (G4), and (H1) to (H4).