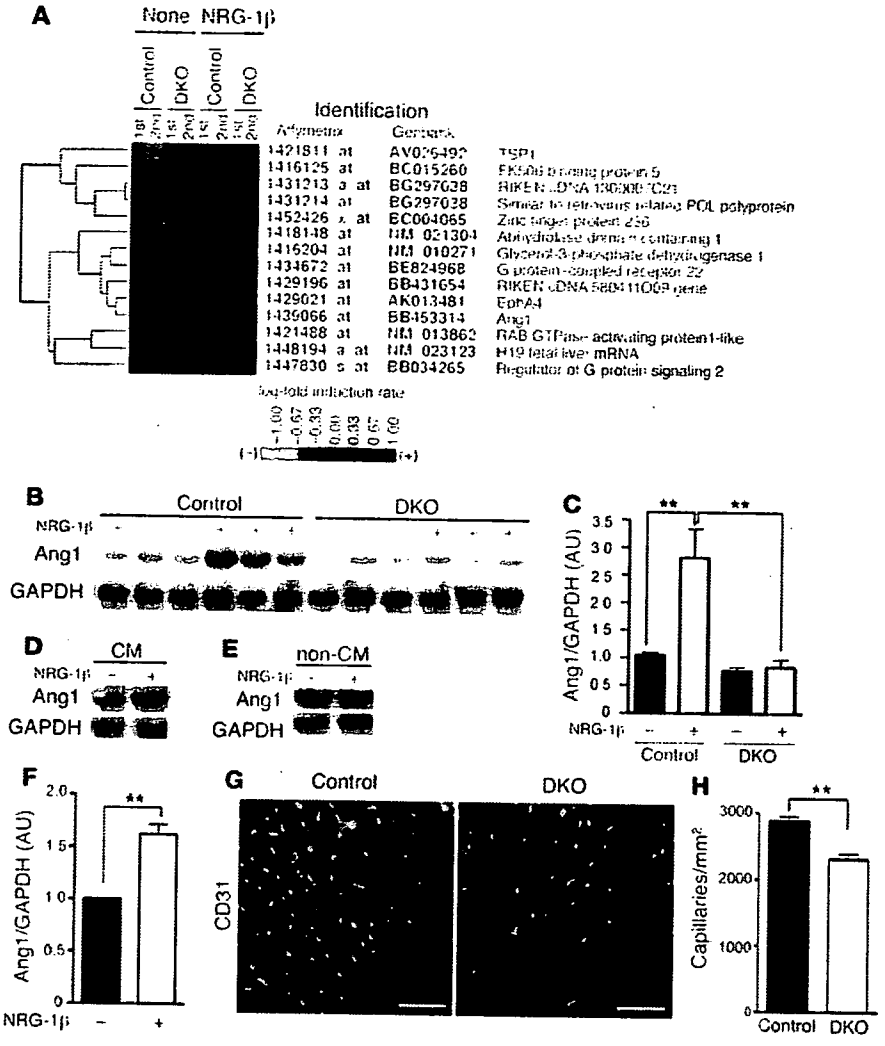

Figure 8

Gab1 and Gab2 are required for the NRG-1β–induced Ang1 upregulation and endothelial stabilization in the heart. (**A**) RNAs from the ventricles of control and DKO mice (n = 3 per group) were prepared 8 hours after injection with NRG-1β or vehicle. This preparation was performed twice. We used pooled RNAs from 3 mice and performed Affymetrix DNA microarrays independently 2 times (indicated as 1st and 2nd). Cluster analysis was performed of upregulated (red) and downregulated (green) genes in NRG-1β–treated control and DKO mice. Color intensity is relative to the median (black). (**B**) Northern blot analysis demonstrated the upregulation of *Ang1* mRNA in the hearts of control mice but not in those of DKO mice following injection with NRG-1β (n = 3 per group). *GAPDH* mRNA was checked for gel loading. (**C**) Quantitative analysis of *Ang1* mRNA (normalized to *GAPDH* mRNA) (n = 3 per group; $^{**}P < 0.01$ between the indicated groups). (**D** and **E**) Ang1 expression was upregulated in cardiomyocytes but not in noncardiomyocytes after stimulation with NRG-1β. (**F**) Quantitative analysis of *Ang1* mRNA normalized to *GAPDH* mRNA (n = 4; $^{**}P < 0.01$). (**G**) Cryosections of the hearts from control and DKO mice were immunostained with anti-CD31 Ab. Representative results are shown. Scale bars: 50 μm. (**H**) Capillary densities were counted from the number of capillaries per square millimeter. $^{**}P < 0.01$.

Biochemical analyses. Mice (body weight, approximately 20 g) were injected with following agonists: LIF (1×10^4 U), NRG-1β (5 μg), HB-EGF (5 μg), EGF (5 μg), FGF2 (2 μg), IGF-1 (5 μg), HGF (2 μg), PDGF-BB (5 μg), and erythropoietin (600 IU). Briefly, mice anesthetized by avertin were injected i.v. via the inferior vena cava with various growth factors and cytokines dissolved in 100 μl of normal saline. The hearts were isolated 5 minutes after injection and washed with ice-cold 4°C PBS. After removing both atria, the ventricles were snap-frozen in liquid nitrogen. The ventricles were homogenized in lysis buffer containing 50 mM HEPES, 100 mM sodium fluoride, 2 mM sodium orthovanadate, 4 mM EDTA, 1% Tween-20, 0.1% SDS, and a protease inhibitor cocktail Complete (Roche Applied Science) using a polytron homogenizer as described previously (50). The lysates were cleared by centrifugation at 17,000 *g* for 30 minutes. Protein concentration was measured with BCA protein assay kit (Pierce). Lysates of cultured cells were prepared essentially as described previously (13). After stimulation, cells were immediately lysed in ice-cold lysis buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 1 mM sodium vanadate, 1 mM dithiothreitol, and a protease inhibitor cocktail Complete). The cleared lysates were subjected to IP and IB following standard procedures as described previously (13).

Creation of a conditional Gab1 mutant allele. Genomic DNA fragments of *Gab1* were isolated from a λ-FixII 129/Sv mouse strain genomic library, and a targeting construct was engineered using a triple-*loxP* system (provided by W. Reith, Department of Pathology and Immunology, University of Geneva Medical School, Geneva, Switzerland). We flanked exon 2, which encodes part of pleckstrin homology domain with 2 *loxP* sites (which codes for amino acids 25–123). The linearized construct DNA was introduced into R1 embryonic stem cells by electroporation. G418-resistant cell colonies were screened for homologous recombination by PCR, as reconfirmed by Southern blot analysis. Embryonic stem cell clones with a *loxP*-floxed exon 2 ($Gab1^{flox}$) without the TK-neo cassette were obtained by transient transfection with a pCre-Pac (provided by T. Yagi, Osaka University) (51). The engineered embryonic stem cells were injected into C57BL/6J blastocysts.

Generation of Gab1CKO and DKO mice. $Gab1^{flox}$ allele was generated in 129/Sv-C57BL/6J mixed background. The transgenic mice expressing Cre recombinase under the control of α-MHC promoter in C57BL/6J background (α-MHC-Cre mice) were generated as previously reported (35). $Gab1^{flox\,flox}$ mice were crossed with α-MHC-Cre mice to generate Gab1CKO mice ($Gab1^{flox\,flox}Gab2^{+/+}$α-*MHC-Cre(+)*). We had also previously created Gab2KO mice and reported that these mice are viable and display defects in mast cells (9, 11). We further crossed Gab1CKO mice with Gab2KO mice to create DKO mice ($Gab1^{flox\,flox}Gab2^{-/-}$ α-*MHC-Cre(+)*). PCR primers used for mouse genotyping are listed in the supplemental information. All mice

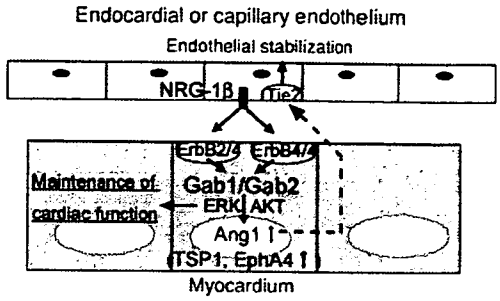

Figure 9
Schematic illustration of the roles of Gab family proteins in the myocardium. NRG-1β shed from the endocardial or capillary endothelium in the heart activates ErbB receptors on the myocardium, resulting in tyrosine phosphorylation of Gab family proteins and subsequent activation of ERK and AKT. NRG-1β/ErbB–Gab1/Gab2 signaling in the myocardium is directly required for postnatal maintenance of myocardial function. In addition, NRG-1β/ErbB–Gab1/Gab2 signaling indirectly contributes to postnatal stabilization of capillary or endocardial endothelium, possibly through Ang1 upregulation (dotted line).

were maintained on a 129/Sv-C57BL/6J mixed background. We housed all animals in a virus-free facility on a 12-hour light/12-hour dark cycle and fed them a standard mouse food. Animal experiments were approved by the National Cardiovascular Center Research Committee and were performed according to the institutional guidelines.

Histological analyses. Hearts from mice at indicated ages (see Results and Figure 3 legend) were fixed with 10% neutralized formalin, embedded in paraffin, and sectioned at 4 μm thickness. Masson trichrome and elastica van Gieson staining were performed on serial sections. To examine the capillary density, hearts of 4- to 6-week-old mice were fixed with 4% paraformaldehyde, cryoprotected with 20% sucrose, and frozen in OCT compound (Sakura). Cryosections (10 μm) were stained with rat monoclonal anti-CD31 Ab and FITC-conjugated secondary Ab. Paraffin sections of hearts from 4- to 6-week-old mice were immunostained with anti-vWF or anti-α-SMA Ab from the EnVision+ Kit (Dako) according to the manufacturer's instructions.

Physiological analyses. For echocardiography, male mice at the indicated ages were anesthetized with 2.5% avertin (Wako; 15 μl/g body weight). Echocardiography was performed using a Hewlett Packard Sonos 5500 Echocardiography System and a 15-MHz linear transducer. Ventricular dimensions were measured on M-mode images at least 3 times for each group of mice. For hemodynamic measurements, 12-week-old male mice were anesthetized with i.p. injection of urethane (750 mg/kg) and α-chloralose (50 mg/kg) dissolved in normal saline (52). The right common carotid artery was exposed via the midline incision. To allow the use of a physiologic closed-chest preparation, the LV was catheterized retrogradely from the carotid artery using a high-fidelity pressure transducer catheter (1.4 French; Millar Instruments) (53). The LV pressure was digitized, stored on the hard disk of a dedicated laboratory computer system, and analyzed with custom software.

Northern blot analysis. The probes for ANP, α-SKA, and GAPDH were kindly donated by K.R. Chien (Massachusetts General Hospital, Boston, Massachusetts, USA). Total RNA was prepared by TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNAs from the ventricles of mouse hearts and cultured cardiomyocytes were subjected to Northern blot analyses against a panel of cDNA probes indicated in Results and the legends for Figures 6 and 8. Hybridization was performed using Quikhyb (Stratagene). Quantitative data were collected after normalizing the results to GAPDH.

Gene expression profiling using Affymetrix DNA microarrays. Gene expression in cardiac ventricular tissue was analyzed by Affymetrix microarray hybridization. Control and DKO male mice at 6 weeks of age were injected with 5 μg of NRG-1β or vehicle (normal saline) via tail vein. The ventricles were isolated at 8 hours after injection and subjected to extraction of total RNA using TRIzol reagent. We pooled total RNA from 3 mouse ventricles for each group and subjected to the microarray hybridization as described previously (54). Preparation of cRNA and hybridization of probe on arrays were performed according to the manufacturer's instructions (Affymetrix). Each array experiment was performed in duplicate. FileMaker Pro 8.0 software was used to analyze genes that demonstrated identical patterns in 2 independent microarray experiments. Data were analyzed according to the minimum information about a microarray experiment (MIAME) rule. Annotation of the probe numbers and targeted sequences are shown on the Affymetrix website (https://www.affymetrix.com/site/login/login.affx).

Statistics. All data are expressed as mean ± SEM. Differences among multiple groups were compared by 1-way ANOVA followed by a post-hoc comparison using Scheffe's method. The 2-tailed Student's *t* test was used to analyze differences between 2 groups. $P < 0.05$ was considered statistically significant. Survival curves were generated using Kaplan-Meier method, and significance was evaluated using the log-rank test.

Acknowledgments

This work was supported in part by grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (to Y. Nakaoka and N. Mochizuki); the Ministry of Health, Labour, and Welfare of Japan (to N. Mochizuki); the Program for the Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (to T. Minami, T. Kodama, and N. Mochizuki); the Japan Heart Foundation (Novartis Grant for Research Award on Molecular and Cellular Cardiology, to Y. Nakaoka); the Mitsubishi Pharma Research Foundation (to Y. Nakaoka); and the Uehara Memorial Foundation (to Y. Nakaoka). We thank Y. Matsuura, M. Yoshida, M. Miyabayashi, M. Maeoka, Y. Ohba, M. Sato, K. Sako, and K. Shioya for their technical assistance; S. Higashiyama, O. Nakagawa, and O. Ohara for helpful comments; K. Komamura for advice on echocardiography; and W.W. Hall and K. Yamauchi-Takihara for critical reading of the manuscript.

Received for publication October 16, 2006, and accepted in revised form April 10, 2007.

Address correspondence to: Naoki Mochizuki, Department of Structural Analysis, National Cardiovascular Center Research Institute, 5-7-1 Fujishirodai, Suita, Osaka 565-8565, Japan. Phone: 81-6-6833-5012, ext. 2508; Fax: 81-6-6835-5461; E-mail: nmochizu@ri.ncvc.go.jp. Or to: Yoshikazu Nakaoka: Department of Cardiovascular Medicine, Osaka University Graduate School of Medicine, 2-2, Yamada-oka, Suita, Osaka 565-0871, Japan. Phone: 81-6-6879-3835; Fax: 81-6-6879-3839; E-mail: ynakaoka@imed3.med.osaka-u.ac.jp.

Y. Nakaoka's present address is: Department of Cardiovascular Medicine, Osaka University Graduate School of Medicine, Osaka, Japan.

Hisao Hirota is deceased.

1. Seidman, J.G., and Seidman, C. 2001. The genetic basis for cardiomyopathy: from mutation identification to mechanistic paradigms. *Cell.* **104**:557–567.
2. Chien, K.R. 1999. Stress pathways and heart failure. *Cell.* **98**:555–558.
3. Schlessinger, J., and Lemmon, M.A. 2003. SH2 and PTB domains in tyrosine kinase signaling. *Sci. STKE.* **2003**:RE12.
4. Gu, H., and Neel, B.G. 2003. The "Gab" in signal transduction. *Trends Cell Biol.* **13**:122–130.
5. Nishida, K., and Hirano, T. 2003. The role of Gab family scaffolding adapter proteins in the signal transduction of cytokine and growth factor receptors. *Cancer Sci.* **94**:1029–1033.
6. Itoh, M., et al. 2000. Role of Gab1 in heart, placenta, and skin development and growth factor- and cytokine-induced extracellular signal-regulated kinase mitogen-activated protein kinase activation. *Mol. Cell. Biol.* **20**:3695–3704.
7. Sachs, M., et al. 2000. Essential role of Gab1 for signaling by the c-Met receptor in vivo. *J. Cell Biol.* **150**:1375–1384.
8. Gu, H., et al. 2001. Essential role for Gab2 in the allergic response. *Nature.* **412**:186–190.
9. Nishida, K., et al. 2002. Requirement of Gab2 for mast cell development and KitL/c-Kit signaling. *Blood.* **99**:1866–1869.
10. Wada, T., et al. 2005. The molecular scaffold Gab2 is a crucial component of RANK signaling and osteoclastogenesis. *Nat. Med.* **11**:394–399.
11. Nishida, K., et al. 2005. FcepsilonRI-mediated mast cell degranulation requires calcium-independent microtubule-dependent translocation of granules to the plasma membrane. *J. Cell Biol.* **170**:115–126.
12. Seiffert, M., et al. 2003. Gab3-deficient mice exhibit normal development and hematopoiesis and are immunocompetent. *Mol. Cell. Biol.* **23**:2415–2424.
13. Nakaoka, Y., et al. 2003. Activation of gp130 transduces hypertrophic signal through interaction of scaffolding/docking protein Gab1 with tyrosine phosphatase SHP2 in cardiomyocytes. *Circ. Res.* **93**:221–229.
14. Bentires-Alj, M., et al. 2006. A role for the scaffolding adapter GAB2 in breast cancer. *Nat. Med.* **12**:114–121.
15. Yamasaki, S., et al. 2003. Gab1 is required for EGF receptor signaling and the transformation by activated ErbB2. *Oncogene.* **22**:1546–1556.
16. Brutsaert, D.L. 2003. Cardiac endothelial-myocardial signaling: its role in cardiac growth, contractile performance, and rhythmicity. *Physiol. Rev.* **83**:59–115.
17. Garratt, A.N., Ozcelik, C., and Birchmeier, C. 2003. ErbB2 pathways in heart and neural diseases. *Trends Cardiovasc. Med.* **13**:80–86.
18. Falls, D.L. 2003. Neuregulins: functions, forms, and signaling strategies. *Exp. Cell Res.* **284**:14–30.
19. Iwamoto, R., and Mekada, E. 2006. ErbB and HB-EGF signaling in heart development and function. *Cell Struct. Funct.* **31**:1–14.
20. Lemmens, K., Segers, V.F., Demolder, M., and De Keulenaer, G.W. 2006. Role of neuregulin-1/ErbB2 signaling in endothelium-cardiomyocyte crosstalk. *J. Biol. Chem.* **281**:19469–19477.
21. Zhao, Y.Y., et al. 1998. Neuregulins promote survival and growth of cardiac myocytes. Persistence of ErbB2 and ErbB4 expression in neonatal and adult ventricular myocytes. *J. Biol. Chem.* **273**:10261–10269.
22. Gassmann, M., et al. 1995. Aberrant neural and cardiac development in mice lacking the ErbB4 neuregulin receptor. *Nature.* **378**:390–394.
23. Lee, K.F., et al. 1995. Requirement for neuregulin receptor erbB2 in neural and cardiac development. *Nature.* **378**:394–398.
24. Meyer, D., and Birchmeier, C. 1995. Multiple essential functions of neuregulin in development. *Nature.* **378**:386–390.
25. Iwamoto, R., et al. 2003. Heparin-binding EGF-like growth factor and ErbB signaling is essential for heart function. *Proc. Natl. Acad. Sci. U. S. A.* **100**:3221–3226.
26. Jackson, L.F., et al. 2003. Defective valvulogenesis in HB-EGF and TACE-null mice is associated with aberrant BMP signaling. *EMBO J.* **22**:2704–2716.
27. Slamon, D.J., et al. 2001. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N. Engl. J. Med.* **344**:783–792.
28. Suter, T.M., Cook-Bruns, N., and Barton, C. 2004. Cardiotoxicity associated with trastuzumab (Herceptin) therapy in the treatment of metastatic breast cancer. *Breast.* **13**:173–183.
29. Crone, S.A., et al. 2002. ErbB2 is essential in the prevention of dilated cardiomyopathy. *Nat. Med.* **8**:459–465.
30. Garcia-Rivello, H., et al. 2005. Dilated cardiomyopathy in Erb-b4-deficient ventricular muscle. *Am. J. Physiol. Heart Circ. Physiol.* **289**:H1153–H1160.
31. Ozcelik, C., et al. 2002. Conditional mutation of the ErbB2 (HER2) receptor in cardiomyocytes leads to dilated cardiomyopathy. *Proc. Natl. Acad. Sci. U. S. A.* **99**:8880–8885.
32. Nakagawa, O., et al. 1995. Rapid transcriptional activation and early mRNA turnover of brain natriuretic peptide in cardiocyte hypertrophy. Evidence for brain natriuretic peptide as an "emergency" cardiac hormone against ventricular overload. *J. Clin. Invest.* **96**:1280–1287.
33. Cote, G.M., Miller, T.A., Lebrasseur, N.K., Kuramochi, Y., and Sawyer, D.B. 2005. Neuregulin-1alpha and beta isoform expression in cardiac microvascular endothelial cells and function in cardiac myocytes in vitro. *Exp. Cell Res.* **311**:135–146.
34. Agah, R., et al. 1997. Gene recombination in postmitotic cells. Targeted expression of Cre recombinase provokes cardiac-restricted, site-specific rearrangement in adult ventricular muscle in vivo. *J. Clin. Invest.* **100**:169–179.
35. Yamaguchi, O., et al. 2004. Cardiac-specific disruption of the c-raf-1 gene induces cardiac dysfunction and apoptosis. *J. Clin. Invest.* **114**:937–943. doi:10.1172/JCI200420317.
36. Meng, S., Chen, Z., Munoz-Antonia, T., and Wu, J. 2005. Participation of both Gab1 and Gab2 in the activation of the ERK/MAPK pathway by epidermal growth factor. *Biochem. J.* **391**:143–151.
37. Moss, A.J., and Adams, F.H. 1995. *Moss' heart disease in infants, children, and adolescents.* Williams & Wilkins. Baltimore, Maryland, USA. 1,085 pp.
38. Westwood, M., Harris, R., Burn, J.L., and Barson, A.J. 1975. Heredity in primary endocardial fibroelastosis. *Br. Heart J.* **37**:1077–1084.
39. Xu, X., et al. 2005. ASF/SF2-regulated CaMKIIdelta alternative splicing temporally reprograms excitation-contraction coupling in cardiac muscle. *Cell.* **120**:59–72.
40. Brindle, N.P., Saharinen, P., and Alitalo, K. 2006. Signaling and functions of angiopoietin-1 in vascular protection. *Circ. Res.* **98**:1014–1023.
41. Davis, S., et al. 1996. Isolation of angiopoietin-1, a ligand for the TIE2 receptor, by secretion-trap expression cloning. *Cell.* **87**:1161–1169.
42. Suri, C., et al. 1996. Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. *Cell.* **87**:1171–1180.
43. Baliga, R.R., et al. 1999. NRG-1-induced cardiomyocyte hypertrophy. Role of PI-3-kinase, p70(S6K), and MEK-MAPK-RSK. *Am. J. Physiol.* **277**:H2026–H2037.
44. Lawler, J. 2000. The functions of thrombospondin-1 and-2. *Curr. Opin. Cell Biol.* **12**:634–640.
45. Ogita, H., et al. 2003. EphA4-mediated Rho activation via Vsm-RhoGEF expressed specifically in vascular smooth muscle cells. *Circ. Res.* **93**:23–31.
46. Liao, W., et al. 1997. The zebrafish gene cloche acts upstream of a flk-1 homologue to regulate endothelial cell differentiation. *Development.* **124**:381–389.
47. Nebigil, C.G., et al. 2000. Serotonin 2B receptor is required for heart development. *Proc. Natl. Acad. Sci. U. S. A.* **97**:9508–9513.
48. Sato, T.N., et al. 1995. Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. *Nature.* **376**:70–74.
49. Nishida, K., et al. 1999. Gab-family adapter proteins act downstream of cytokine and growth factor receptors and T- and B-cell antigen receptors. *Blood.* **93**:1809–1816.
50. Osugi, T., et al. 2002. Cardiac-specific activation of signal transducer and activator of transcription 3 promotes vascular formation in the heart. *J. Biol. Chem.* **277**:6676–6681.
51. Taniguchi, M., et al. 1998. Efficient production of Cre-mediated site-directed recombinants through the utilization of the puromycin resistance gene, pac: a transient gene-integration marker for ES cells. *Nucleic Acids Res.* **26**:679–680.
52. Georgakopoulos, D., et al. 1998. In vivo murine left ventricular pressure-volume relations by miniaturized conductance micromanometry. *Am. J. Physiol.* **274**:H1416–H1422.
53. Nishio, R., Sasayama, S., and Matsumori, A. 2002. Left ventricular pressure-volume relationship in a murine model of congestive heart failure due to acute viral myocarditis. *J. Am. Coll. Cardiol.* **40**:1506–1514.
54. Minami, T., Miura, M., Aird, W.C., and Kodama, T. 2006. Thrombin-induced autoinhibitory factor, Down syndrome critical region-1, attenuates NFAT-dependent vascular cell adhesion molecule-1 expression and inflammation in the endothelium. *J. Biol. Chem.* **281**:20503–20520.