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Table S1

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LRP: Role in Vascular Wall Integrity and Protection from Atherosclerosis

Philippe Boucher,[1]* Michael Gotthardt,[1]* Wei-Ping Li,[2] Richard G. W. Anderson,[2] Joachim Herz[1]†

Vascular smooth muscle cell (SMC) proliferation and migration are important events in the development of atherosclerosis. The low-density lipoprotein receptor–related protein (LRP1) mediates suppression of SMC migration induced by platelet-derived growth factor (PDGF). Here we show that LRP1 forms a complex with the PDGF receptor (PDGFR). Inactivation of LRP1 in vascular SMCs of mice causes PDGFR overexpression and abnormal activation of PDGFR signaling, resulting in disruption of the elastic layer, SMC proliferation, aneurysm formation, and marked susceptibility to cholesterol-induced atherosclerosis. The development of these abnormalities was reduced by treatment with Gleevec, an inhibitor of PDGF signaling. Thus, LRP1 has a pivotal role in protecting vascular wall integrity and preventing atherosclerosis by controlling PDGFR activation.

Blood vessels must resist the stress of constant pounding and shear forces of flowing blood. Vascular wall integrity is necessary to prevent aneurysmal dilatation and rupture (*1*), and elevated plasma cholesterol levels lead to cholesterol infiltration into the wall and decrease its stability. Factors that control vascular integrity include collagen (*2*), elastin (*3*, *4*), and proteases and their inhibitors (*5*, *6*), as well as growth factors such as platelet-derived growth factor (PDGF), which causes smooth muscle cell (SMC) proliferation at sites of stress (*7*, *8*). PDGF induces SMC migration in vitro and this activity can be blocked by binding of apolipoprotein E (ApoE) to low-density lipoprotein (LDL) receptor–related protein-1 (LRP1) (*9*–*11*). LRP1 is a multifunctional protein that binds a variety of biologically diverse ligands (*12*). Tyrosine phosphorylation of LRP1 occurs in response to PDGF, requires the PDGF receptor β (PDGFRβ) and the phosphatidylinositol-3 kinase and is blocked by ApoE (*13*, *14*). Thus, a role of LRP1 may be to limit the activity of signals elicited by PDGF and possibly other growth factors. To test whether LRP1 could be involved in controlling SMC proliferation, an important step in atherosclerotic lesion development and progression, we generated tissue-specific knockout mice that lack LRP1 only in vascular SMCs.

We achieved smooth muscle–specific LRP1 (smLRP) inactivation by crossing SM22Cre transgenic mice (*15*) with LRP^{flox} animals (*16*). In contrast to conventional LRP knockouts (*17*), $SM22Cre^{+}$;$LRP^{flox/flox}$ ($smLRP^{-}$) mice were born alive and appeared superficially normal. To increase susceptibility to spontaneous atherosclerotic lesion development, these animals were crossed to LDL receptor knockout ($LDLR^{-}$) mice to generate $LDLR^{-}$;$smLRP^{-}$ mice. The $LDLR^{-}$ mouse is an excellent model for studying human atherosclerosis, because atherosclerotic lesion formation can be accelerated and experimentally controlled over a wide range by cholesterol feeding (*18*).

The presence or absence of LRP1 expression in SMCs had no effect on plasma cholesterol or triglyceride levels, in mice on normal chow or an atherogenic high-cholesterol diet (fig. S1) (*19*). However, aortas from $smLRP^{-}$ mice were consistently distended and dilated (Fig. 1A). This difference increased over time and was accompanied by thickening of the aortic wall (Fig. 1A, b), pronounced atherosclerosis (Fig. 1A, c, arrows), and aneurysm formation (Fig. 1A, e). Matrix metalloproteinase activity (MMP2 and MMP9) was modestly increased in the aortas of $LDLR^{-}$;$smLRP^{-}$ mice (fig. S1). Both proteinases are ligands for LRP, and increased MMP2 expression has been found to correlate with abdominal aneurysm formation in humans (*6*). In the $smLRP^{-}$ mice, MMP2 and MMP9 accumulation in the vessel wall may be secondary because of reduced receptor-mediated clearance, increased tissue remodeling, or both.

The increased number of cells with typical flat nuclei in aortas from $smLRP^{-}$ mice suggests that the aortic thickening was primarily caused by SMC proliferation (Fig. 1B). LRP immunoreactivity was virtually absent in aortas of $LDLR^{-}$;$smLRP^{-}$ mice (Fig. 1B, d) indicating the efficiency of SMC-specific gene inactivation. Immunohistochemistry of the normal vessel wall shows that LRP1 was expressed in SMCs (Fig. 1B, c). The elastic laminae between the SMCs were grossly disrupted in the $smLRP^{-}$ vessel wall (Fig. 1B, f). These findings suggest a role of LRP1 in the concerted assembly and restructuring of the elastic and SMC layers. Vessel wall thickening progressed with age (Fig. 1B, g to l), resulting in almost complete occlusion of mesenteric arteries in $smLRP^{-}$ animals.

This increased tissue proliferation and restructuring may be the cause for the greatly increased sensitivity of $smLRP^{-}$ animals to cholesterol-induced atherosclerosis when compared to $smLRP^{+}$ controls (Fig. 1A, d to h). Preparations of $LDLR^{-}$;$smLRP^{-}$ mouse hearts and aortas extending to the iliac bifurcation show substantial lengthening, dilatation, and thickening of the unopened vessels (Fig. 1A, d

[1]Department of Molecular Genetics, [2]Department of Cell Biology, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, TX 75390–9046, USA.

*These authors contributed equally to this work.
†To whom correspondence should be addressed. E-mail: Joachim.Herz@UTSouthwestern.edu

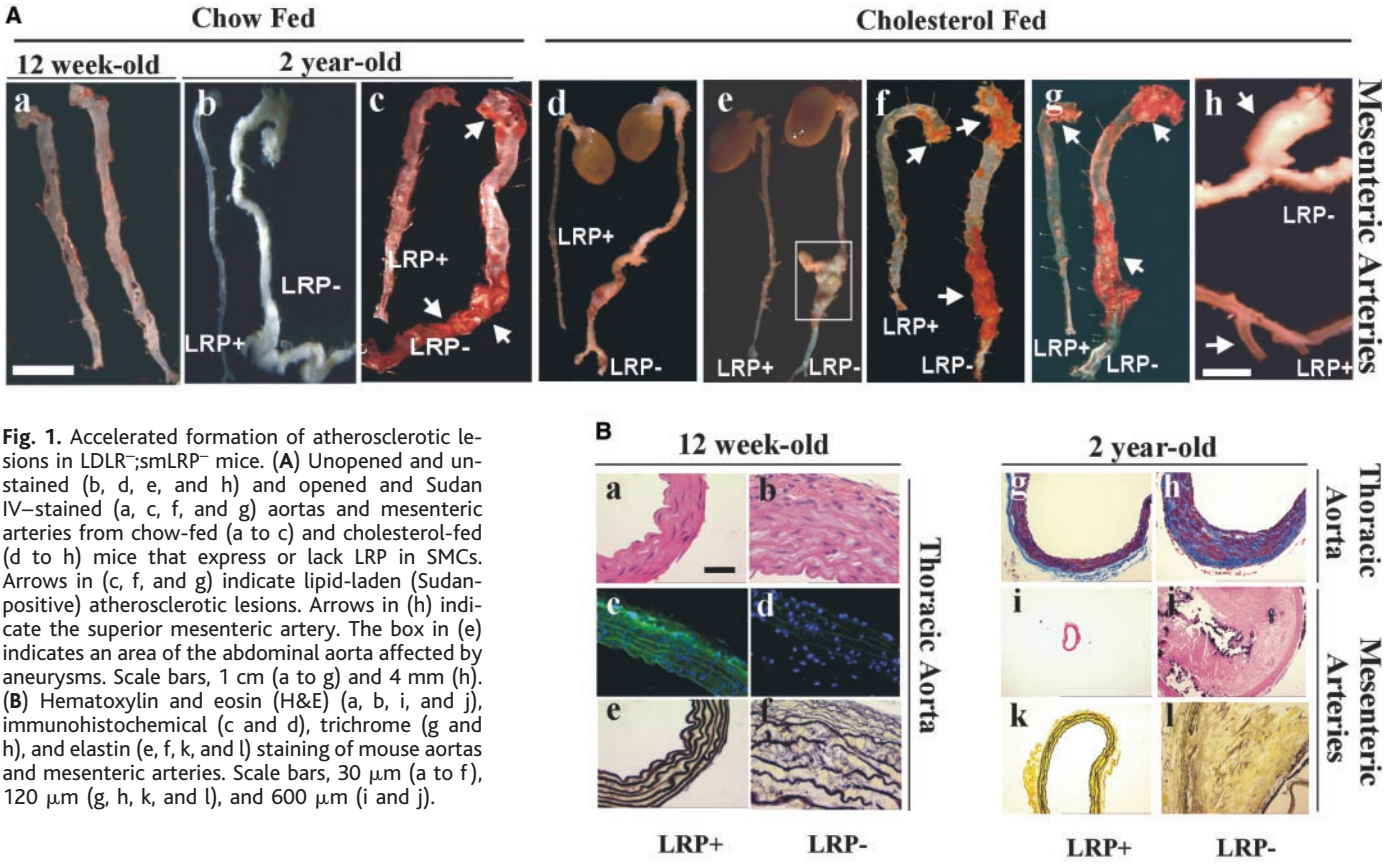

Fig. 1. Accelerated formation of atherosclerotic lesions in LDLR^{-};smLRP^{-} mice. (**A**) Unopened and unstained (b, d, e, and h) and opened and Sudan IV–stained (a, c, f, and g) aortas and mesenteric arteries from chow-fed (a to c) and cholesterol-fed (d to h) mice that express or lack LRP in SMCs. Arrows in (c, f, and g) indicate lipid-laden (Sudan-positive) atherosclerotic lesions. Arrows in (h) indicate the superior mesenteric artery. The box in (e) indicates an area of the abdominal aorta affected by aneurysms. Scale bars, 1 cm (a to g) and 4 mm (h). (**B**) Hematoxylin and eosin (H&E) (a, b, i, and j), immunohistochemical (c and d), trichrome (g and h), and elastin (e, f, k, and l) staining of mouse aortas and mesenteric arteries. Scale bars, 30 μm (a to f), 120 μm (g, h, k, and l), and 600 μm (i and j).

and e) with large aneurysms (Fig. 1A, e, boxed area). Prominent atherosclerotic lesions were revealed by Sudan IV staining of the lumenal surface of the same longitudinally dissected aortas. Arrows (Fig. 1A, f and g) indicate predominantly affected locations in the aortic arch and in the abdominal aorta around the branch points of the renal and mesenteric arteries (Fig. 1A, h). Histology of smLRP^{-} aortas after cholesterol feeding also revealed gross thickening of the vessel wall, which was caused by a combination of cellular proliferation, foam cell transformation, and cholesterol deposition in interstitial clefts (fig. S2).

LRP1 undergoes tyrosine phosphorylation on its cytoplasmic domain in response to the growth factor PDGF-bb (*13*, *14*), which in turn promotes binding of the Shc adaptor protein (*20*). PDGF-bb is a potent inducer of SMC migration and proliferation. Exposure of SMCs in culture to ApoE-containing lipoproteins abrogates the stimulatory effect of PDGF-bb on cell migration (*9*–*11*), but partial knockdown of LRP1 expression prevented this inhibitory effect of ApoE. Furthermore, PDGF-bb is a ligand for LRP1 (*13*), and ApoE-containing lipoproteins prevent the PDGF-induced tyrosine phosphorylation of the LRP1 cytoplasmic domain (*14*). PDGF and other growth factors also associate with α2-macroglobulin, an abundant proteinase inhibitor that becomes a ligand for LRP1 upon binding of a proteinase and is thus removed from the extracellular space by LRP1-mediated endocytosis (*12*).

Taken together, these findings raise the possibility that phosphorylation of the LRP1 tail in response to PDGF might play a role in regulating cellular growth and migration. To test this hypothesis, we used the tyrosine kinase inhibitor STI571 (Gleevec), a compound that was initially developed with the goal of preventing restenosis after coronary angioplasty through inhibition of abnormal PDGFR activation. Gleevec also inhibits several other tyrosine kinases, and it is now used clinically with great success to treat several forms of malignancies (*21*).

We first ascertained that Gleevec suppresses LRP1 phosphorylation in response to PDGF-bb in the cultured bovine vascular SMC line CRL 20-18. Gleevec completely blocked the PDGF-induced tyrosine phosphorylation of the LRP1 cytoplasmic domain (Fig. 2A), as well as phosphorylation of PDGFRβ, which coprecipitated with LRP1 as a tyrosine-phosphorylated 190-kD protein (p190) from PDGF-bb treated cells. PDGFRβ activity also coimmunoprecipitated with LRP1 from untreated cell lysates. Tyrosine phosphorylation of LRP1 and PDGFRβ could be achieved by treatment of the isolated immune complexes with PDGF-bb in vitro (Fig. 2A, right lanes).

To test whether Gleevec could prevent cholesterol-induced atherosclerotic lesion progression in LDLR^{-};smLRP^{-} animals in vivo, the drug was mixed into the cholesterol diet at a calculated dose of ~10 mg per kg of body weight per day (mg/kg/day) and animals were fed this diet for 6 weeks. Gleevec had a profound protective effect and greatly reduced the area and size of atherosclerotic lesions in the abdominal aortas of LDLR^{-};smLRP^{-} mice and partially reduced lesions in the aortic arch, the area that in the mouse is most susceptible to atherosclerosis (*22*) (Fig. 2, B and C). The few lesions that appeared in LDLR^{-};smLRP^{+} aortas on this feeding regime were also reduced upon Gleevec treatment. Furthermore, Gleevec treatment reduced vascular wall thickening and foam cell formation and improved the stability of the elastic layer in the aortas and mesenteric arteries of LDLR^{-};smLRP^{-} mice (Fig. 2D).

Because Gleevec can inhibit several other tyrosine kinases (*21*), these data alone do not establish that PDGFRβ is functionally deregulated in smLRP^{-} mice. To investigate whether PDGFRβ signaling is indeed increased in these animals, we prepared aorta extracts from chow-fed LDLR^{-};smLRP^{+} and LDLR^{-};smLRP^{-} mice. The extracts were analyzed by Western blotting for the presence of total PDGFRβ, tyrosine-phosphorylated PDGFRβ, phospho-Erk (activated by PDGF signaling), and LRP1. In the absence of LRP1, PDGFRβ expression and tyrosine phos-

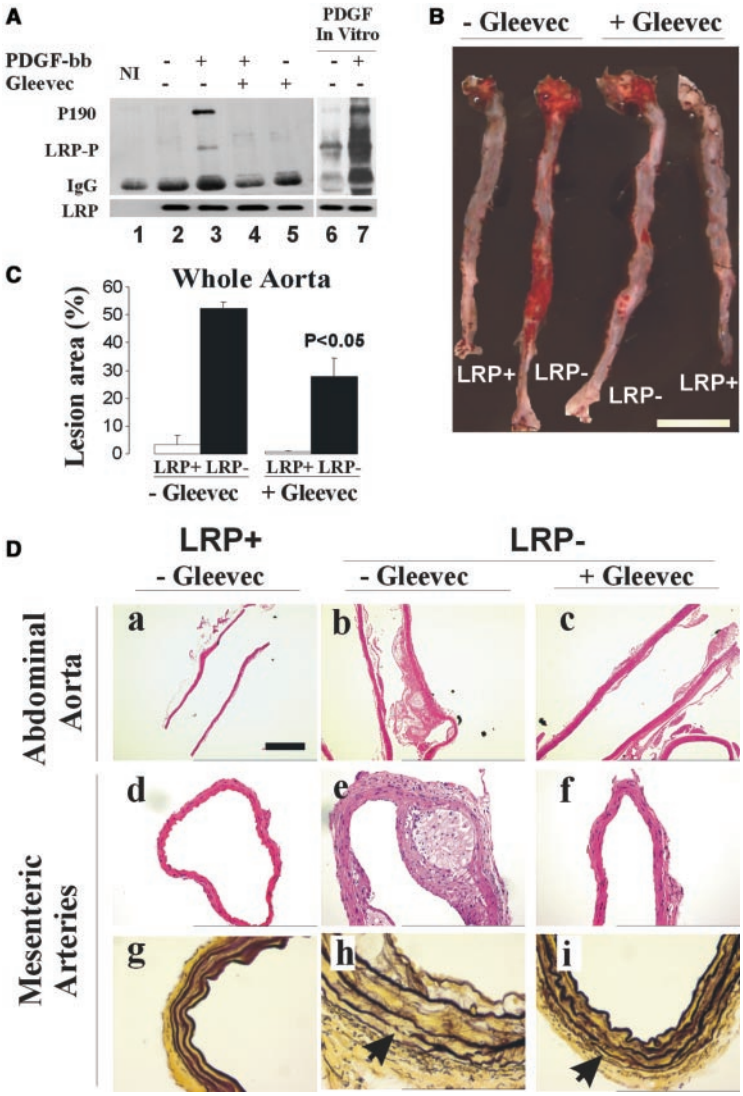

Fig. 2. Effect of Gleevec on atherosclerotic lesion formation, vascular wall integrity, and LRP1 tyrosine phosphorylation. (**A**) Effect of Gleevec on PDGF-induced tyrosine phosphorylation of LRP1 in the bovine vascular SMC line CRL 20-18. Serum-starved cells were incubated in the absence (lanes 1, 2, and 5) or presence (lanes 3 and 4) of PDFG-bb (90 ng/ml) and in the absence (lanes 1 to 3) or presence (lanes 4 and 5) of 1 μM Gleevec for 15 min. Proteins were immunoprecipitated from lysates with anti-LRP1 or nonimmune (NI) immunoglobulin G and immunoblotted with anti-phosphotyrosine (top) or antibodies to LRP (bottom). Lanes 6 and 7, anti-LRP immune complexes from untreated cells, were treated for 15 min with PDGF-bb in vitro and immunoblotted for phosphotyrosine (top) or LRP (bottom). LRP-P, tyrosine phosphorylation of the LRP1 cytoplasmic domain. (**B**) Sudan IV–stained aortas from 20-week-old LDLR^{-};smLRP^{-} (LRP^{-}) and control LDLR^{-};smLRP^{+} (LRP^{+}) mice that had been cholesterol-fed for 6 weeks in the absence (–) or presence (+) of Gleevec (Novartis, 10 mg/kg/day) before analysis. Scale bar, 1 cm. (**C**) Quantitative analysis of atherosclerotic lesion size in aortas from cholesterol-fed mice (n = 6 mice per group) with and without Gleevec treatment. Error bars, SEM. (**D**) Histological analysis of lesions in abdominal aortas and mesenteric arteries of cholesterol-fed animals in the absence and presence of Gleevec. Longitudinal (a to c) and transverse (d to i) sections were stained with H&E (a to f) or van Gieson's stain for elastin (g to i). Gleevec treatment markedly reduced vascular wall thickness, prevented lesion formation, and improved elastic lamina integrity (indicated by the arrows) in LRP^{-} animals. Scale bars, 600 μm (a to c), 120 μm (d to f), and 30 μm (g to i).

phorylation (activation) were markedly increased, resulting in activation of Erk1/2, a downstream event of PDGF signaling.

Immunohistochemistry of LRP1-expressing and LRP1-deficient aortas revealed that this activation of the PDGFRβ signaling cascade occurred exclusively in SMCs (Fig. 3). These vessels had not yet developed atherosclerotic lesions, as determined by the absence of foam cells, cholesterol deposits, or infiltration by inflammatory cells. Activation of PDGFRβ signaling in LRP1^{-} SMCs thus precedes atherosclerotic lesion formation. This finding is consistent with our hypothesis that deregulated PDGFRβ signaling is an important component of the increased atherosclerosis susceptibility in the absence of smooth muscle LRP1.

Our experiments in smLRP^{-} mice have revealed a pivotal role of LRP1 in protecting the integrity of the vascular wall. Although the somatic gene defect we have engineered in this animal model would be unlikely to occur in this form in humans, the underlying mechanism nevertheless further emphasizes the importance of PDGF signaling for human atherosclerosis.

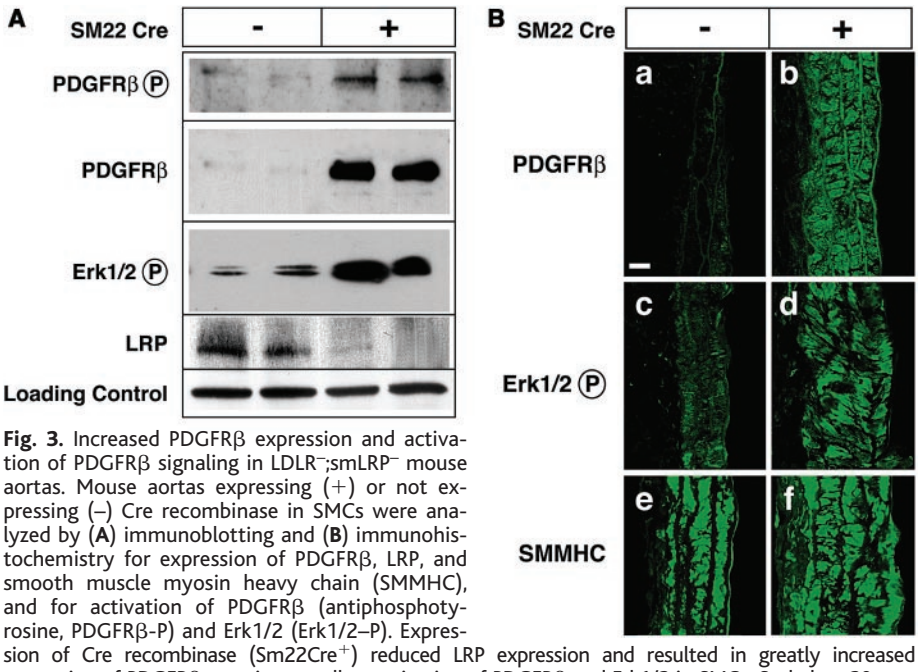

Fig. 3. Increased PDGFRβ expression and activation of PDGFRβ signaling in LDLR^{-};smLRP^{-} mouse aortas. Mouse aortas expressing (+) or not expressing (–) Cre recombinase in SMCs were analyzed by (**A**) immunoblotting and (**B**) immunohistochemistry for expression of PDGFRβ, LRP, and smooth muscle myosin heavy chain (SMMHC), and for activation of PDGFRβ (antiphosphotyrosine, PDGFRβ-P) and Erk1/2 (Erk1/2–P). Expression of Cre recombinase (Sm22Cre^{+}) reduced LRP expression and resulted in greatly increased expression of PDGFRβ protein, as well as activation of PDGFRβ and Erk1/2 in SMCs. Scale bar, 20 μm.

In contrast to the role of LRP1 in the liver (*23*), direct cellular lipoprotein uptake is apparently not involved. Rather, this atheroprotective effect of LRP1 in the vessel wall seems to be due mainly to its role in controlling PDGFR-dependent signaling pathways and other mechanisms that increase SMC proliferation and migration (fig. S3)—events that accelerate the progression of atherosclerotic lesions in the presence of predisposing risk factors, such as elevated plasma cholesterol.

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Supporting Online Material

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Role of the *Arabidopsis* Glucose Sensor HXK1 in Nutrient, Light, and Hormonal Signaling

Brandon Moore,*†, Li Zhou,* Filip Rolland, Qi Hall, Wan-Hsing Cheng,‡ Yan-Xia Liu, Ildoo Hwang,§ Tamara Jones, Jen Sheen‖

Glucose modulates many vital processes in photosynthetic plants. Analyses of *Arabidopsis glucose insensitive2* (*gin2*) mutants define the physiological functions of a specific hexokinase (HXK1) in the plant glucose-signaling network. HXK1 coordinates intrinsic signals with extrinsic light intensity. HXK1 mutants lacking catalytic activity still support various signaling functions in gene expression, cell proliferation, root and inflorescence growth, and leaf expansion and senescence, thus demonstrating the uncoupling of glucose signaling from glucose metabolism. The *gin2* mutants are also insensitive to auxin and hypersensitive to cytokinin. Plants use HXK as a glucose sensor to interrelate nutrient, light, and hormone signaling networks for controlling growth and development in response to the changing environment.

Glucose is a universal nutrient preferred by most organisms and serves fundamental roles in energy supply, carbon storage, biosynthesis, and carbon skeleton and cell wall formation. The ability to sense glucose signals is important for organisms as diverse as *Escherichia coli*, yeast, flies, mammals, and plants (*1*–*5*). In contrast to the extensive information available on glucose sensing and signaling mechanisms in bacteria and unicellular eukaryotes (*1*, *3*), the importance of glucose as a direct and central signaling molecule in multicellular animals and plants has only recently been recognized (*2*, *4*, *5*). In plants whose lives revolve around sugar production through photosynthesis, glucose has emerged as a key regulator of many vital processes, including germination; seedling development; root, stem, and shoot growth; photosynthesis; carbon and nitrogen metabolism; flowering; stress responses; and senescence. (*4*). However, it remains unclear how plants integrate environmental factors and internal sugar signals to modulate growth and development.

In *E. coli*, yeast, mammals, and plants, hexokinases (HXKs) and other sugar kinases are the most ancient and evolutionarily conserved sugar sensors (*1*, *3*, *4*). The *Arabidopsis* genome encodes two *HXK* and four *HXK*-like (*HKL*) genes (*6*–*8*). To determine the precise physiological functions of HXKs in plant growth and development, we designed a two-step genetic screen to isolate loss-of-function HXK mutants in *Arabidopsis*. We first screened for *glucose insensitive* (*gin*) mutants that overcome development arrest on a 6% glucose Murashige and Skoog (MS) medium (*6*, *9*). We then used a specific HXK antibody to identify two putative HXK mutants (*gin2-1* and *gin2-2*) (Fig. 1A). The mutations were mapped to chromosome IV between the cleaved amplified polymorphic sequences marker RPS2 and the simple sequence length polymorphism marker nga 1139, where *HXK1* is located (*6*). Both *gin2* mutants are recessive (table S1). DNA cloning and sequencing identified the molecular lesions of these two HXK1 mutants: a nonsense mutation (Q432*) in *gin2-1* and a missense mutation (G416A) in *gin2-2* (Fig. 1B). The result was consistent with reduced *HXK1* but not *HXK2* transcripts in *gin2-1* (Fig. 1C), because nonsense mutations often destabilize mRNA. The predicted truncated HXK1 protein was below detectable levels in *gin2-1*, indicating that this is a HXK1 null mutant (Fig. 1D). The antibody to HXK detected transiently expressed *Arabidopsis* HXK1 and HXK2 proteins equally well (Fig. 1D) (*10*), but HXK2 protein did not accumulate. Low HXK2 protein accumulation also occurred in a *35S::HXK2* transgenic line (*6*) (Fig. 1D). The *gin2-1* mutant was complemented by a *35S::HXK1* transgene on a 6% glucose MS medium (Fig. 1E). The glucose-insensitive phenotype displayed by the *gin2* mutants indicated that endogenous HXK2 and HKL expression cannot compensate for the loss of HXK1 function in the seedling glucose response assay. Subsequent experiments were mostly carried out with *gin2-1*.

To unravel the physiological functions of HXK1 in glucose sensing without the use of exogenous glucose, we grew wild-type and *gin2-1* plants under different light conditions in a growth chamber. High light intensity can boost photosynthesis, sugar production, and plausibly glucose signaling in a physiological

Department of Genetics, Harvard Medical School, and Department of Molecular Biology, Massachusetts General Hospital, Boston, MA 02114, USA.

*These authors contributed equally to this work.
†Present address: Department of Genetics and Biochemistry, Clemson University, 100 Jordan Hall, Clemson, SC 29631, USA.
‡Present address: Botany Institute, Academia Sinica, Taipei, Taiwan.
§Present address: Department of Life Science, Pohang University of Science and Technology, Pohang 790-784, Korea.
‖To whom correspondence should be addressed. E-mail: sheen@molbio.mgh.harvard.edu