Interaction between Connexin50 and Mitogen-activated Protein Kinase Signaling in Lens Homeostasis

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Submitted January 2, 2009; Revised March 13, 2009; Accepted March 13, 2009

Monitoring Editor: Benjamin Margolis

Both connexins and signal transduction pathways have been independently shown to play critical roles in lens homeostasis, but little is known about potential cooperation between these two intercellular communication systems. To investigate whether growth factor signaling and gap junctional communication interact during the development of lens homeostasis, we examined the effect of mitogen-activated protein kinase (MAPK) signaling on coupling mediated by specific lens connexins by using a combination of *in vitro* and *in vivo* assays. Activation of MAPK signaling pathways significantly increased coupling provided by Cx50, but not Cx46, in paired *Xenopus laevis* oocytes *in vitro*, as well as between freshly isolated lens cells *in vivo*. Constitutively active MAPK signaling caused macrophthalmia, cataract, glucose accumulation, vacuole formation in differentiating fibers, and lens rupture *in vivo*. The specific removal or replacement of Cx50, but not Cx46, ameliorated all five pathological conditions in transgenic mice. These results indicate that MAPK signaling specifically modulates coupling mediated by Cx50 and that gap junctional communication and signal transduction pathways may interact in osmotic regulation during postnatal fiber development.

INTRODUCTION

Multicellular organisms require regulated communication between cells for the normal growth and differentiation of complex organs. Such intercellular communication can be directly mediated between cells by the intercellular channels present in gap junctions or indirectly propagated by extracellular growth factors that bind receptors to activate signal transduction cascades. Both gap junctions and signal transduction pathways have been shown to play critical roles in the development of the ocular lens (Gerido and White, 2004; Lovicu and McAvoy, 2005; Robinson, 2006; Gong *et al.*, 2007), but to date only a few studies have addressed the critical question of whether there is any interaction between these two different modes of intercellular communication (Le and Musil, 2001; Boswell *et al.*, 2008a,b).

The lens is composed of three cell types: a monolayer of epithelial cells covering the anterior surface, differentiating fiber cells in the lens cortex, and mature fibers that fill the lens core (Piatigorsky, 1981). All three cell types are directly coupled to their neighbors by gap junctions containing three distinct connexins (Goodenough, 1992; Mathias *et al.*, 1997). Cx43 is only present in the lens epithelium, Cx46 is expressed only in the differentiating and mature fiber cells, and Cx50 is found throughout both the epithelium and lens fibers (Beyer *et al.*, 1989; Musil *et al.*, 1990; Paul *et al.*, 1991; White *et al.*, 1992, 2007). Cx50 knockout animals exhibit

significantly reduced postnatal mitosis, decreased lens and eye growth, delayed fiber denucleation, and mild cataracts (White *et al.*, 1998; Rong *et al.*, 2002; Sellitto *et al.*, 2004). It has also been observed that mice bearing point mutations in Cx50 show profound defects in the differentiation of both primary and secondary fiber cells (Xia *et al.*, 2006). These data have clearly established that intercellular communication mediated by Cx50 can contribute to the regulation of epithelial cell proliferation and fiber cell differentiation during lens development (Gong *et al.*, 2007).

In addition to the regulation imposed by gap junctional coupling, it has long been known that lens development is also strongly influenced by growth factor signaling. For example, fibroblast growth factors (FGFs) and fibroblast growth factor receptors (FGFRs) regulate lens induction, epithelial cell proliferation and fiber differentiation (Robinson *et al.*, 1995; Lovicu and McAvoy, 2001; Zhao *et al.*, 2008). Consistent with these observations, lens-specific expression of a dominant-negative form of Ras reduces epithelial cell proliferation and delays fiber elongation (Xie *et al.*, 2006). Although the defects in cell proliferation and differentiation that result from experimental manipulation of mitogen-activated protein kinase (MAPK) signaling are often more profound than those that arise after genetic mutation of the lens connexins, there is some overlap in the range of observed developmental deficiencies.

Epithelial cells differentiate into secondary fibers at the lens equator, in which high levels of MAPK signaling are stimulated by growth factors, including FGF (Le and Musil, 2001; Lovicu and McAvoy, 2005). Differentiating fiber cells at the lens equator also have the highest measured levels of gap junctional coupling compared with either the anterior and posterior poles, or the lens core (Baldo and Mathias, 1992; Mathias *et al.*, 2007). This high coupling at the equator

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E08-12-1257>) on March 25, 2009.

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is critical in the establishment of an internal circulating current that drives the nonvascular microcirculatory system used by the lens to maintain transparency (Mathias *et al.*, 1997; Donaldson *et al.*, 2001). Previous *in vitro* work has shown that this developmentally regulated increase in junctional coupling is dependent on cross-talk between the FGF and bone morphogenetic protein signaling pathways (Le and Musil, 2001; Boswell *et al.*, 2008a,b). Although these results support the notion that equatorial FGF signaling interacts with lens connexins to produce the observed asymmetry in gap junctional coupling, they have failed to identify the specific connexins that can interact with MAPK signaling *in vivo*.

In the current study, we have investigated the mechanisms whereby gap junctional coupling and MAPK signaling may interact in the establishment of postnatal lens homeostasis. We have examined the functional interaction of lens connexins with MAPK signaling *in vitro* in paired *Xenopus* oocytes and *in vivo* in genetically engineered mice. We have found that activation of MAPK signaling significantly increased coupling provided by Cx50 but not Cx46 *in vitro*. Constitutively active MAPK signaling also specifically increased Cx50 functional activity between lens cells *in vivo* and produced macrophthalmia, cataracts, an increase in glucose accumulation, the formation of vacuoles in the bow region of the lens fibers, and lens rupture. The specific removal of Cx50, but not Cx46, as a potential target of constitutive MAPK signaling ameliorated all of these lens pathologies in mice. These results indicate that MAPK signaling specifically modulates Cx50, but not Cx46, and that Cx50-mediated gap junctional communication and signal transduction pathways may work together in the establishment of osmotic homeostasis during postnatal fiber development.

MATERIALS AND METHODS

In Vitro Transcription, Oocyte Microinjection, and Pairing

Cx50, Cx46, *Xenopus* FGF receptor, and constitutively active mitogen-activated protein kinase kinase (MEK) 1(E) coding sequences were subcloned into pCS2+, linearized, and used as the template to produce cRNAs by using the SP6 mMessage mMachine (Ambion, Austin, TX). Oocytes were isolated from *Xenopus laevis* frogs (Nasco, Fort Atkinson, WI), defolliculated by collagenase and hyaluronidase digestion, and cultured in modified Barth's (MB), or oocyte Ringer 3, medium. Cells were injected with 40 nl of an antisense oligonucleotide (3 ng/cell) to suppress the endogenous *Xenopus* Cx38 or a mixture of antisense plus cRNA (40 ng/cell) by using a Nanoject II injector (Drummond, Broomall, PA) and allowed to recover for 24 h. To pair cells, oocytes were immersed in hypertonic solution to strip the vitelline envelope, transferred to a Petri dish containing MB medium, and manually paired with vegetal poles apposed. Electrophysiological recordings were made 12–16 h after pairing. For experiments testing MEK1(E), cells were injected with MEK1(E) 12–16 h after injection with connexin cRNA. For experiments testing FGF, 12 h after pairing cells were incubated with 15 ng/ml basic FGF ligand (Sigma-Aldrich, St. Louis, MO) for 2 h and then recorded. In some experiments, the MEK inhibitor U0126 (DuPont, Wilmington, DE) was added 30 min before FGF ligand.

Dual Whole-Cell Voltage Clamp

Cell-to-cell channels were assessed by dual voltage clamp (Spray *et al.*, 1981). Current and voltage electrodes (1.2 mm in diameter, omega dot; Glass Company of America, Millville, NJ) were pulled to a resistance of 1–2 M Ω with a vertical puller (Narishige, Tokyo, Japan) and filled with 3 M KCl, 10 mM EGTA, and 10 mM HEPES, pH 7.4. Voltage clamping of oocyte pairs was performed using two GeneClamp 500 amplifiers (Axon Instruments, Foster City, CA) controlled by a PC-compatible computer using Digidata 1320A interface (Axon Instruments). pCLAMP software (Axon Instruments) was used to program the stimulus and data collection paradigms. For measurements of junctional coupling (G_j), both cells in the pair were clamped at -40 mV to eliminate any transjunctional potential (V_j). Cell one was then subjected to alternating pulses of ± 20 mV while the current produced by the change in voltage was recorded in cell two. The current delivered to the

second cell was equal in magnitude to the junctional current (I_j), and (G_j) was calculated by dividing the measured current by the voltage difference as follows: $G_j = I_j / (V_1 - V_2)$, where V_1 and V_2 are the voltages in the first and second cells, respectively.

Preparation of Oocyte Samples for Western Blot

For soluble proteins, oocytes were collected in 10 μ l of lysis buffer per oocyte containing 137 mM NaCl, 20 mM Tris, pH 8.0, 2 mM EDTA, 1% IGEPAL (Sigma-Aldrich), 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich) and 0.5 μ M Na₃VO₄ and homogenized by pipetting oocytes up and down. Samples were then centrifuged at $14,000 \times g$ for 10 min at 4°C. The supernatants were collected and supplemented with an equal volume of 2 $\times$ SDS sample buffer, separated on 15% SDS-polyacrylamide gel electrophoresis (PAGE) gels, and transferred to nitrocellulose membranes. For membrane proteins, oocytes were collected in 1 ml of ice-cold lysis buffer containing 5 mM Tris, pH 8.0, 5 mM EDTA, and protease inhibitors and homogenized using a series of mechanical passages through syringe needles of diminishing diameter (20, 22, and 26 Ga). Extracts were centrifuged at $1000 \times g$ at 4°C for 5 min. The supernatants were then centrifuged at $100,000 \times g$ at 4°C for 30 min. Membrane pellets were resuspended in SDS sample buffer (2 μ l/oocyte), separated on 10% SDS-PAGE gels, and transferred to nitrocellulose membranes. Membrane protein blots were probed with polyclonal Cx50 or Cx46 rabbit antibodies (Paul *et al.*, 1991; White *et al.*, 1992) at a 1:1000 dilution followed by incubation with alkaline phosphatase-conjugated anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). Soluble protein blots were probed with rabbit polyclonal phospho (p)-extracellular signal-regulated kinase (ERK) or total ERK antibodies (Cell Signaling Technology, Danvers, MA.) at 1:1000 dilution, followed by incubation with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

Mouse Breeding

MEK1(E) transgenic mice have lenses that express a constitutively active mutant of MEK1 under the α A-crystallin promoter (Gong *et al.*, 2001) and were interbred with Cx50KO (White *et al.*, 1998), Cx46KO (Gong *et al.*, 1997), or Cx50KI46 mice (White, 2002) to produce MEK1(E)-Cx50KO, MEK1(E)-Cx46KO, or MEK1(E)-Cx50KI46 animals. Genomic DNAs isolated from tail biopsies were genotyped by polymerase chain reaction (PCR) (Gong *et al.*, 1997, 2001; White *et al.*, 1998; White, 2002).

Lens Epithelial Cell Isolation

Lenses were dissected from eyes and transferred to calcium- and magnesium-free phosphate-buffered saline (PBS). The lens capsule was then peeled away from the fiber cell mass by using fine forceps. For Western blotting, capsules were transferred to 2 $\times$ sample buffer (15 μ l/lens), separated on SDS-PAGE gels, and transferred to nitrocellulose membranes. Blots were probed with polyclonal Cx50, Cx46, Cx43, p-ERK, or total ERK rabbit antibodies followed by either alkaline phosphatase-conjugated, or HRP-conjugated, anti-rabbit secondary antibodies. For electrophysiology, epithelial cells were dissociated by incubation of capsules with 0.25% trypsin for 5–7 min at 37°C and plated onto glass coverslips for electrophysiological studies. Junctional currents were measured 1–4 h after dissociation (White *et al.*, 2007).

Dual Whole-Cell Patch Clamp

Junctional conductance was measured between cell pairs by using dual whole-cell patch clamp with Axopatch 1D patch-clamp amplifiers (Axon Instruments) at room temperature. The solution bathing the cells contained 135 mM NaCl, 5 mM KCl, 2 mM CsCl, 2 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES, 5 mM dextrose, 2 mM pyruvate, and 1 mM BaCl₂, pH 7.4. Patch electrodes had resistances of 3–5 M Ω when filled with internal solution containing 125 mM CsCl, 10 mM EGTA, 0.5 mM CaCl₂, and 10 mM HEPES, pH 7.2. Macroscopic and single-channel recordings were acquired by using PCLAMP8 software (Axon Instruments), sampled at 1–2 kHz and filtered at 0.2–0.5 kHz, and analysis was performed with PCLAMP8 and ORIGIN 6.0 software (MicroCal Software, Northampton, MA). Each cell of a pair was initially held at a common holding potential of 0 mV. To evaluate junctional coupling, 200-ms hyperpolarizing pulses from the holding potential of 0 to -20 mV were applied to one cell to establish a transjunctional voltage gradient (V_j), and junctional current was measured in the second cell (held at 0 mV). Quinine (Sigma-Aldrich) was used to selectively block Cx50 conductance because its effects are fully and rapidly reversible, and it has no effect on Cx46 or Cx43 junctional currents (Srinivas *et al.*, 2001; Cruikshank *et al.*, 2004; Bai *et al.*, 2006). Quinine solutions were applied with a gravity-fed perfusion system, and solution exchange was complete within 10 s. The magnitude of inhibition caused by quinine is expressed as the percentage of the conductance inhibited by the drug.

5-Bromo-2'-deoxyuridine (BrdU) Injection

Postnatal mouse pups were injected intraperitoneally with 100 μ g/g body weight of BrdU (Sigma-Aldrich). BrdU at 10 mg/ml was dissolved in PBS at 37°C just before use. Injected pups were returned to their mothers for 1 h

incubation, euthanized, and lenses were dissected. The lens capsule was then peeled away from the fiber cell mass by using fine forceps and pinned down on an encapsulant (Sygard; Dow Corning, Midland, MI) coated 35-mm Petri dish and fixed for 30 min in 2% formaldehyde in PBS. Fixed capsules were rinsed with PBS, incubated with 100% MeOH at -20°C for 5 min, mounted on microscope slides, and allowed to air dry. BrdU was immunolabeled with an in situ detection kit (BD Biosciences Pharmingen, San Diego, CA) according to the manufacturer's instructions, with the exception that endogenous peroxidase was quenched with 0.3% hydrogen peroxide diluted in absolute methanol, and all antibody incubations were carried at 37°C . BrdU-negative nuclei were counterstained with aqueous hematoxylin (Sellitto *et al.*, 2004). Stained sections were viewed and photographed with a digital camera (MagnaFire; Optonics, Goleta, CA).

Quantitation of BrdU Labeling

The diameters of BrdU-labeled capsules were individually measured for each genotype at each developmental age (P2 or P5). A rectangular area with a length of three-fifths diameter and a height of one-fifth diameter was drawn for each capsule. The rectangle was positioned to cover the area from the lens equator to the center of the lens. BrdU-positive nuclei were counted within the rectangular region and divided by the area to calculate the density of BrdU labeled cells. Statistical analysis between wild-type, MEK1(E), Cx50KO, and MEK1(E)-Cx50KO lenses was performed using the three-way analysis of variance (ANOVA).

Growth Analysis and Lens Photography

Eyes of age-matched male animals were dissected, blotted dry on tissue paper, and individually weighed. Eyes were transferred to a Petri dish containing 37°C M199 medium (Sigma-Aldrich) with 10 mM HEPES, pH 7.4, on a warm stage. Lenses were dissected and transferred to a prewarmed Petri dish with a glass bottom (WPI, Sarasota, FL) filled with M199 medium. Lenses were visualized and photographed through a SZX9 dissecting microscope equipped with a C3030 zoom digital camera (Olympus of America, Lake Success, NY), blotted dry on tissue paper, and individually weighed (Martinez-Wittinghan *et al.*, 2003).

Histology

Mouse eyes were dissected and fixed in a 4% formaldehyde solution in PBS for 16–24 h at room temperature. Fixed eyes were rinsed with PBS, dehydrated through an ethanol series, and embedded in paraffin. Sections of 2–3 μm were cut on a diamond knife, deparaffinized, and stained with hematoxylin-eosin. Histological sections were viewed on a BX51 microscope (Olym-

pus of America) and photographed with a digital camera (MagnaFire; Optonics).

Glucose Concentration Measurement

Six lenses from 3-wk-old wild-type, Cx50KO, MEK1(E), and MEK1(E)-Cx50KO lenses were dissected and pooled for each measurement. Lenses were deproteinized by homogenization with a 5-mm Polytron (Brinkmann Instruments, Westbury, NY) in 300 μl of 6% HClO_4 solution, followed by centrifugation at $18,000 \times g$ for 10 min at 4°C . Supernatants were neutralized with 70 μl of 2 M K_2CO_3 and centrifuged at $18,000 \times g$ for 10 min at 4°C . Glucose was quantified by the Glucose (HK) Assay kit (Sigma-Aldrich) according to the manufacturer's instructions. Lens glucose concentration was calculated from the total glucose amount per lens and lens volume. Lens volume was calculated assuming the lens were a sphere based on $1/6 \pi D^3$, using the equatorial diameter (D).

RESULTS

MEK1 Activity Increases Cx50 Junctional Conductance in Paired Oocytes

If MAPK signaling interacts with gap junctional coupling in the development of lens homeostasis, then activation of the MAPK pathway may have specific functional effects on intercellular channels composed of lens connexins. To test this idea, we coexpressed a constitutively active form of MEK1 kinase, MEK1(E), with either Cx46, or Cx50, in an in vitro paired *Xenopus* oocyte assay and measured junctional conductance (G_j). As shown in Figure 1, injection of oocyte pairs with either water or MEK1(E) alone failed to induce appreciable cell-to-cell coupling. Pairs expressing Cx50 were well coupled with a mean G_j of 6.1 μS . Oocytes coexpressing Cx50 with MEK1(E) displayed a 3.4-fold increase in gap junctional coupling ($p < 0.05$, Student's t test) over pairs containing Cx50 alone (Figure 1A). This significant enhancement of Cx50-mediated junctional conductance could have resulted from either an increase in the level of connexin protein, or from the activation of quiescent Cx50 channels by MAPK signaling. To distinguish between these possibilities,

Figure 1. MAPK signaling specifically increases Cx50 junctional conductance. (A) Junctional conductance measurements recorded from *Xenopus* oocyte pairs injected with Cx50 and MEK1(E) transcript alone or in combination. Cell pairs expressing water or MEK1(E) did not form functional intercellular channels. Cx50 pairs had a mean conductance value of $\sim 6 \mu\text{S}$. Coexpression of MEK1(E) with Cx50 significantly increased conductance ($\sim 21 \mu\text{S}$; $p < 0.05$, Student's t test). Immunoblot analysis of oocytes showed equivalent protein levels of wild-type Cx50 (B) and total endogenous ERK (C). (D) Expression of MEK1(E) stimulated phosphorylation of ERK. (E) Junctional conductance measurements recorded from *Xenopus* oocyte pairs injected with wild-type Cx46 and MEK1(E) transcript alone or in combination. Cx46 and Cx46+MEK1(E) coexpressing pairs formed gap junctions with mean conductances of equal value ($p > 0.05$, Student's t test). Immunoblot analysis of oocytes showed equivalent levels of wild-type Cx46 (F) and total ERK protein expression for the conditions tested (G). (H) Expression of MEK1(E)-stimulated ERK phosphorylation.

homogenates were prepared from oocytes injected with water, MEK1(E), Cx50, or Cx50 and MEK1(E) and analyzed for protein expression by Western blotting. When blots were probed with an anti-Cx50 antibody, qualitatively similar levels of an ~60-kDa band corresponding to Cx50 were visualized in cells injected with Cx50 cRNA alone or in combination with MEK1(E). No signal was detected from cells receiving water or MEK1(E) alone (Figure 1B). To examine MAPK signaling, Western blots were probed with anti-total-ERK and anti-p-ERK antibodies. Injection of the constitutively active MEK1(E) cRNA did not alter the endogenous levels of total ERK (Figure 1C). However, MEK1(E) expression activated the MAPK pathway in oocytes as shown by the large increase in phosphorylated (p)-ERK detection in cells that were injected with MEK1(E) alone or in combination with Cx50 (Figure 1D). Together, these data suggest that Cx50 and MEK1(E) functionally interact in vitro and that the augmentation of Cx50 mediated conductance was not due to an increase in connexin protein expression but was directly correlated with ERK phosphorylation.

Cx46 Does Not Functionally Interact with MEK1(E)

We have previously shown that the in vivo replacement of Cx50 with Cx46 by genetic knockin was not sufficient to restore defects in lens development caused by Cx50 knockout (White *et al.*, 1998; White, 2002; Rong *et al.*, 2002). If interactions between Cx50 and MAPK signaling are required for normal development, then part of the failure of Cx46 to substitute for Cx50 could be caused by an inability to functionally interact with MAPK signaling. To examine this possibility, we repeated our oocyte experiments using Cx46 cRNA (Figure 1E). As expected, oocytes injected with water or MEK1(E) alone were uncoupled and cell pairs expressing Cx46 alone were robustly coupled, with a mean G_j of 10.8 μS . In contrast to the Cx50 data, oocyte pairs that coexpressed Cx46 with MEK1(E) had conductance values that were not significantly different from the pairs expressing Cx46 alone ($G_j = 12.5 \mu S$; $p > 0.05$). Analysis of Cx46 expression (Figure 1F) confirmed qualitatively equivalent protein levels in oocytes injected either with Cx46 alone or in combination with MEK1(E). Examination of total and phosphorylated ERK showed that the expression of MEK1(E) greatly increased p-ERK levels in oocytes, whether or not they coexpressed Cx46 (Figure 1H). These data imply that coupling mediated by Cx46 is not affected by MEK1(E) and that there is a connexin specific interaction between MAPK signaling and Cx50.

FGF Receptor Activation Also Increases Cx50-mediated Coupling

MEK1 is the penultimate kinase in the canonical MAPK signaling pathway, and our data show that a constitutively active form, MEK1(E), specifically increases coupling mediated by Cx50 but not Cx46. During lens development, the MAPK signaling pathway is normally activated by the binding of growth factors to their specific receptors. FGF receptor signaling is known to play a key role in lens development (Robinson, 2006) and mediates many of its effects through the MAPK pathway (Lovicu and McAvoy, 2005), leading us to predict that activation of the FGFR may also have an effect on junctional coupling mediated by Cx50. To evaluate this possibility, we coexpressed the *Xenopus* fibroblast growth factor receptor with Cx50 to activate the MAPK signaling pathway via a receptor–ligand interaction. Oocytes were injected with Cx50 and FGFR individually or together and then paired and incubated with 15 ng/ml FGF before ana-

Figure 2. FGFR activation increases Cx50 junctional conductance. (A) Junctional conductance measurements recorded from *Xenopus* oocyte pairs injected with wild-type Cx50 and *Xenopus* FGFR transcripts, followed by incubation with FGF ligand. Cell pairs expressing water or FGFR alone did not develop appreciable conductance. Cx50 conductance was significantly increased (4.6-fold; $p < 0.05$, Student's *t* test) in cell pairs coexpressing FGFR after treatment with FGF. (B) Treatment with the MEK inhibitor U0126 30 min before FGF application blocked the increase in Cx50 coupling in cell pairs coexpressing FGFR.

lyzing intercellular coupling (Figure 2A). Cells expressing the FGFR alone failed to induce cell-to-cell coupling above the levels seen in the water-injected negative control pairs. Oocytes expressing Cx50 alone were coupled at a level >50-fold higher than background, with a mean G_j of 4.6 μS . Cells coexpressing Cx50 with FGFR displayed a 4.6-fold increase in coupling ($p < 0.05$, Student's *t* test) over pairs containing Cx50 alone in the presence of FGF ligand. To test whether MAPK signaling was the mediator of FGFR action, we repeated the experiments in the presence of the MEK inhibitor U0126 (Figure 2B). Addition of 15 μM U0126 30 min before FGF application blocked the FGFR induced increase in Cx50 coupling. These data show that activation of MAPK signaling at the level of receptor–ligand interactions has the same effect on Cx50 mediated communication as coexpression of MEK1(E).

Cx50-mediated Gap Junctional Coupling Is Increased in MEK1(E) Transgenic Lenses

Our in vitro results showed that activation of MAPK signaling by two different approaches significantly increased Cx50-mediated coupling. To examine whether MAPK signaling and Cx50 could also interact in vivo, we analyzed a transgenic mouse model that expressed MEK1(E) in the lens under the control of the αA -crystallin promoter (Gong *et al.*, 2001). Lens fiber cells from MEK1(E) transgenic animals were shown previously to be severely compromised, but their epithelial cells remained intact (Gong *et al.*, 2001). We have previously documented that Cx50 contributes to epithelial cell coupling in a developmentally regulated manner and that its activity can be distinguished from that of Cx43 or Cx46 by the drug quinine, which specifically inhibits Cx50 (Srinivas *et al.*, 2001; White *et al.*, 2007). To test for an interaction between MAPK signaling and Cx50 in vivo, we isolated primary epithelial cell pairs from wild-type and MEK1(E) lenses and measured the quinine sensitive component of their gap junctional conductance. First, postnatal day

Figure 3. MEK1(E) increases ERK phosphorylation and Cx50 coupling in lens epithelial cells. (A) Western blot analyses of capsules from wild-type and MEK1(E) lenses. Total ERK was comparable in wild-type and MEK1(E) lenses, whereas MEK1(E) samples showed an increase in phosphorylated ERK compared with wild-type. (B) Western blotting of Cx43, Cx46, and Cx50 shows that Cx43 and Cx50 are highly expressed in epithelial cells. The absence of Cx46 confirms that the capsule samples were not contaminated by lens fiber material. (C) Perfusion of a P28 wild-type epithelial cell pair with 300 μ M quinine reduced the junctional current (I_j) $\sim$ 25%. (D) In MEK1(E) epithelial cells pairs, the quinine-induced reduction of I_j was $\sim$ 66%. (E) Bar graph summarizing the effect of 300 μ M quinine on coupling in wild-type and MEK1(E) epithelial cells. The percentage of reduction in conductance (i.e., Cx50 contribution) in wild-type P28 cells pairs was 27%, whereas MEK1(E) cell pairs showed a 66% reduction in Cx50 activity. Data represent the mean $\pm$ SE, $n = 8$.

(P) 28 lens capsules, containing the adhering epithelial cell monolayer, were stripped from wild-type and MEK1(E) lenses and analyzed for protein expression by Western blotting. When blots were probed with anti-total-ERK and anti-p-ERK antibodies (Figure 3A), comparable levels of total ERK were seen in both types of lenses. However, an increase in p-ERK was detected in the lens capsules from MEK1(E) transgenic mice. To confirm that the increase in phosphorylated (p)-ERK was coming from the epithelial cell layer and not from potentially contaminating fiber cells, capsule samples were blotted with antibodies to Cx43, Cx46, and Cx50 (Figure 3B). Mouse lens epithelial cells express Cx43 and Cx50 (White *et al.*, 2007), and these two connexins were readily detected. Epithelial cells normally lack Cx46 (Martinez-Wittingham *et al.*, 2003; White *et al.*, 2007), which is normally found at high levels in lens fibers, and no Cx46 signal was detected in our capsule preparations. These data

show that epithelial cells in MEK1(E) transgenic lenses have an elevated level of MAPK signaling.

To test for a MEK1(E) effect on Cx50-mediated coupling, P28 epithelial cells were dispersed into pairs, and junctional currents (I_j) were directly measured by dual whole-cell patch clamp before and after perfusion with 300 μ M quinine, a concentration that reversibly blocks Cx50, but not Cx43, channels (Srinivas *et al.*, 2001). P28 cells were chosen because Cx50 activity is normally minimal at this age (White *et al.*, 2007), and a MEK1(E)-induced increase in Cx50 activity would be easy to distinguish using quinine. Wild-type cell pairs displayed a 25% reduction in I_j when perfused with quinine (Figure 3C), consistent with our previous report that Cx50 is normally a minor contributor to junctional conductance at this developmental age (White *et al.*, 2007). In contrast, P28 MEK1(E) cell pairs had a macroscopic conductance that was twofold higher than wild type, on average, and 66% of the total I_j was quinine sensitive (Figure 3D). If the doubling of magnitude in coupling was due entirely to Cx50 activation by MEK1(E), and there was no Cx43 inactivation, then the Cx43 quinine-insensitive contribution would be expected to drop to 37.5% from 75%, a number in very good agreement with the observed value of 34%. A summary of all perfusion experiments is shown in Figure 3E. Expression of the MEK1(E) transgene increased the quinine-sensitive component of junctional conductance (i.e., Cx50) 2.6-fold ($p < 0.05$), consistent with our *in vitro* studies conducted in *Xenopus* oocytes. Together, these results show that the contribution of Cx50 to lens cell coupling can also be increased by the activation of MAPK signaling *in vivo*.

Postnatal Mitosis Stimulated by the MEK1(E) Transgene Is Diminished in the Absence of Cx50

During normal lens development, Cx50 activity in the epithelium is maximal during peak postnatal mitosis (P2–P3) and declines significantly as the lens matures into an adult organ (P12–P28). In addition, deletion of Cx50 results in a decrease in postnatal mitosis (Sellitto *et al.*, 2004; White *et al.*, 2007). The MEK1(E) transgene significantly increased Cx50 channel activity in the postnatal lens epithelium, maintaining it at a high level as late as P28. To investigate whether this increase in Cx50 coupling correlated with altered mitotic activity in lens capsules, we assayed BrdU incorporation in wild-type, MEK1(E), and MEK1(E)-Cx50KO lenses during the first postnatal week. As shown in Figure 4A, there was no difference in the density of BrdU-labeled cells between wild-type and MEK1(E) lenses on P2, a day when postnatal mitosis and Cx50 activity are normally at their maximum levels (Sellitto *et al.*, 2004; White *et al.*, 2007). Deletion of Cx50 from MEK1(E) lenses reduced BrdU labeling by $\sim$ 30% ($p < 0.05$), consistent with our previous studies (Sellitto *et al.*, 2004; White *et al.*, 2007). On P5, BrdU incorporation was reduced in all lenses compared with P2, but the P5 MEK1(E) transgenic epithelial cells had a density of labeling that was 60% increased over P5 wild type ($p < 0.05$). Deletion of Cx50 from the MEK1(E) animals restored BrdU labeling to wild-type levels on P5 (Figure 4B). In addition to reducing the magnitude of BrdU incorporation on P5, deletion of Cx50 from the MEK1(E) lenses also altered the spatial distribution of mitotic cells. In P5 MEK1(E) lenses, BrdU positive cells were detected across the entire lens capsule (Figure 4C), including the central epithelium (Figure 4D). In contrast, P5 MEK1(E)-Cx50KO capsules had strong labeling in the equatorial “germinative zone” (Figure 4E) but greatly reduced BrdU incorporation in the central epithelium (Figure 4F). These data suggest that MEK1(E) transgene caused a modest

Figure 4. MEK1(E) stimulated mitosis is reduced by Cx50 knock-out. (A) On P2, there were no differences in the density of BrdU-labeled cells between wild-type and MEK1(E) lenses, although deletion of Cx50 from MEK1(E) lenses reduced BrdU labeling by 30%. (B) On P5, the MEK1(E) animals had a density of labeling that was 60% increased over wild-type ($p < 0.05$), and this increase was eliminated by knockout of Cx50 from the MEK1(E) animals. Deletion of Cx50 from the MEK1(E) lenses also altered the spatial distribution of mitotic cells on P5. (C) In P5 MEK1(E) lenses, BrdU-labeled cells were found across the entire capsule, including the central epithelium (D). (E) In P5 MEK1(E)-Cx50KO capsules, BrdU staining was strong in the equatorial zone but greatly reduced in the central epithelium (F).

increase in mitosis on P5 that was dependent on the presence of Cx50 gap junction channels.

Removal of Cx50 Delays Cataract Progression in MEK1(E) Transgenic Lenses

Transgenic overexpression of MEK1(E) in the lens has been reported previously to produce severe cataract (Gong *et al.*, 2001), and our present data showed increased Cx50-mediated coupling in MEK1(E) transgenic lens cells. To examine whether interactions between Cx50 and MAPK signaling contributed to the development of lens opacity in the MEK1(E) transgenic mice, we crossed them with Cx50KO, Cx46KO, or Cx50KI46 animals and monitored the postnatal progression of the cataract phenotype. Wild-type lenses

were transparent between 1 and 7 wk of age (Figure 5, A–D). In contrast, MEK1(E) transgenic lenses contained both nuclear and cortical cataracts at 1 wk (Figure 5E) that progressively worsened to a total lens opacity by 5 wk of age (Figure 5G) and frequently displayed lens rupture by 7 wk (Figure 5H). Deletion of Cx46 from the MEK1(E) transgenic animals had no effect on cataract development (Figure 5, I–L). However, when Cx50 was deleted, the lens showed only a mild nuclear cataract at 1 wk of age (Figure 5M), a phenotype shared by the Cx50 KO lens in the absence of the MEK1(E) transgene (White *et al.*, 1998). At 3 wk, both the central and cortical cataracts were much less severe in the MEK1-Cx50KO lens. By 5 wk, the central cataract was similar to that seen in the MEK1(E) transgenic lens (Figure 5O), but the lens cortex remained cataract free in the MEK1(E)-Cx50KO lens through 7 wk of age (Figure 5P). MEK1(E)-Cx50KI46 lenses, where the coding sequence of Cx50 has been replaced with that of Cx46 (White, 2002), were similar to the MEK1(E)-Cx50KO lenses, with a delay in both the progression and severity of the cataract (Figure 5, Q–T). These results show that the specific removal of Cx50, but not Cx46, can delay cataract onset caused by the constitutive activation of MAPK signaling in the MEK1(E) transgenic lens.

Deletion of Cx50, but Not Cx46, Prevents Rupture in MEK1(E) Lenses

While monitoring cataract progression, we observed that lens rupture frequently accompanied the severe cataract that developed in adult MEK1(E) transgenic mice. To determine whether this process also involved a specific interaction between MAPK signaling and Cx50, we monitored the frequency of lens rupture between 1 and 7 wk for wild-type, MEK1(E), MEK1(E)-Cx46KO, MEK1(E)-Cx50KO, and MEK1(E)-Cx50KI46 transgenic mice. Wild-type lenses never exhibited lens rupture in the time period examined, whereas MEK1(E) lenses began to rupture at 3 wk of age and increased to an incidence of 57% rupture by 7 wk (Figure 6A). Lenses from MEK1(E)-Cx46KO mice were also frequently ruptured, beginning at 3 wk and reaching a level of 45% by 7 wk of age. Interestingly, MEK1(E)-Cx50KO lenses failed to exhibit lens rupture during the 7 wk of observation, and a similar low level of rupture was seen with MEK1(E)-Cx50KI46 lenses (Figure 6B). Together, these data suggest that the deletion, or replacement, of Cx50 as one possible target of MAPK signaling rescued the lens rupture induced by MEK1(E).

Cx50 Is Required for the Development of Macrophthalmia in MEK1(E) Mice

MEK1(E) transgenic animals were shown previously to develop macrophthalmia, with a 32% increase in eye mass and a >40% increase in lens mass compared with wild-type mice (Gong *et al.*, 2001). To determine whether the increase in size of the lens and eye induced by constitutively active MAPK signaling specifically required Cx50, we plotted eye and lens mass as a function of age for wild-type, MEK1(E), connexin knockout, and connexin knockin mice. By 7 wk of age, MEK1(E) eyes were 35% larger than wild-type (Figure 7A), in good agreement with previous data (Gong *et al.*, 2001). Eye growth for Cx46KO mice was indistinguishable from wild-type, whereas Cx50KO and Cx50KI46 mice had reduced eye mass (Figure 7B), as reported previously (Gong *et al.*, 1997; White *et al.*, 1998; White, 2002). Interbreeding MEK1(E) transgenic mice with the different knockin and knockout mice revealed that MEK1(E)-Cx46KO eye growth was identical to that of the MEK1(E) transgenic alone, whereas MEK1(E)-Cx50KO and MEK1(E)-Cx50KI46 were

Figure 5. Delayed cataractogenesis in MEK1(E) lenses lacking Cx50. (A–D) Wild-type lenses from one to seven weeks of age grow normally and remain transparent. (E–F) MEK1(E) lenses developed nuclear and cortical cataracts by 1 wk that progress to total opacity and frequent lens rupture by 7 wk. (I–L) Knockout of Cx46 from MEK1(E) lenses does not alter cataract formation. (M–P) Knockout of Cx50 or replacement of Cx50 with Cx46 (Q–T), results in a slower onset of the central cataract and a reduction in severity of the cortical cataract.

both significantly reduced ($p < 0.05$, ANOVA) from that of the MEK1(E) transgenic (Figure 7C). Lens growth followed a similar pattern, with the MEK1(E) transgene promoting a significant increase in lens mass that was abolished by deletion or replacement of Cx50 but that was unaffected by deletion of Cx46 (Figure 7, D–F). The significant decreases in eye and lens mass for MEK1(E)-Cx50KO and MEK1(E)-Cx50KI46 animals in comparison with MEK1(E) mice sug-

gest that MAPK signaling specifically interacts with Cx50 in the generation of macrophthalmia.

Histological Analysis of Wild-Type, MEK1(E), and MEK1(E)-Cx50KO Lenses

Our data have shown that MEK1(E) increases coupling mediated by Cx50 but not Cx46. We have further documented that the specific removal of Cx50 from MEK1(E) transgenic

Figure 6. Removal of Cx50 from MEK1(E) lenses eliminates rupture. Graphs show the percentage of rupture for wild-type, MEK1(E), MEK1(E)-Cx50KO, MEK1(E)-Cx46KO, and MEK1(E)-Cx50KI46 lenses from 1 to 7 wk of age. (A) Wild-type lenses show no rupture, whereas MEK1(E) lenses exhibit an increasing rate of rupture, reaching 57% at 7 wk of age. (B) Deletion of Cx46 does not alter the incidence of lens rupture. Removal or replacement of Cx50 greatly reduced the incidence of rupture in MEK1(E) lenses.

lenses delays cataractogenesis, eliminates lens rupture, and abolishes macrophthalmia. To gain mechanistic insight into how these observations were possibly linked, we examined histological sections from mouse eyes that were stained with hematoxylin and eosin. On P7, wild-type eyes revealed normal ocular development with an absence of lens pathology in the central and cortical regions (Figure 8, A and D). In contrast, sections of P7 MEK1(E) eyes were characterized by a pronounced loss of eosin staining in the lens nucleus (Figure 8B) and the appearance of numerous vacuoles in the bow region of the lens cortex (Figure 8E), features consistent with the gross morphology of the intact lens at this age (Figure 5E). Sections from MEK1(E)-Cx50KO lenses exhibited a reduced loss of eosinophilic staining in the core of the

lens (Figure 8C) compared with MEK1(E) mice, consistent with the milder cataract seen in intact lenses (Figure 5M). In addition, vacuole formation was never observed in the lens cortex of MEK1(E)-Cx50KO animals (Figure 8F). These data suggest that increased Cx50 coupling induced by MAPK signaling leads to the formation of vacuoles at the lens equator in MEK1(E) transgenic mice. Incorporating these data with previous studies showing that MAPK signaling plays an important role in the increase in gap junctional coupling at the lens equator during fiber differentiation (Mathias *et al.*, 1997; Le and Musil, 2001) further implies that a specific interaction between MAPK signaling and Cx50 may be required for the normal development of lens transport pathways, and that the formation of vacuoles in the MEK1(E) lenses may result from defects in lens transport.

Deletion of Cx50 Reduces MEK1(E)-stimulated Lens Glucose Accumulation in the Lens

An elevation of glucose concentration has been reported previously in MEK1(E) transgenic lenses (Gong *et al.*, 2001). Our lens histology results suggested that removal of Cx50 from the MEK1(E) mice prevented vacuole formation that in turn may have been driven by defects in lens transport. We would predict that if Cx50 deletion ameliorated lens transport defects in MEK1(E) lenses, the previously documented rise in glucose concentration could also be eliminated by Cx50 deletion. To test this possibility, we assayed the glucose concentration in lenses from wild-type, MEK1(E), Cx50KO, and MEK1(E)-Cx50KO mice at 3 wk of age. As shown in Figure 9, the MEK1(E) transgenic lenses exhibited a 60% increase in glucose concentration compared with wild-type. Deletion of Cx50 by itself did not significantly alter the lens glucose concentration from wild-type levels. In contrast, MEK1(E)-Cx50KO lenses were significantly different from the MEK1(E) transgenic alone ($p < 0.05$, Student's *t* test) and restored lens glucose concentration to the wild-type level. These results suggest that the removal of Cx50 in the presence of constitutive MAPK signaling restores lens transport at the equator, which in turn eliminates the formation of vacuoles that are potentially formed by the inappropriate accumulation of solutes like glucose.

DISCUSSION

We have demonstrated specific functional interactions between Cx50 and MAPK signaling in vivo and in vitro.

Figure 7. Deletion or replacement of Cx50, but not Cx46, reverses eye and lens overgrowth caused by MEK1(E). Eye and lens mass were plotted as a function of age. By 7 wk of age, MEK1(E) eyes (A) and lenses (D) were significantly larger than wild-type controls. The eye (B) and lens (E) mass of Cx50KO and Cx50KI46 were smaller than wild-type, whereas Cx46KO organs were indistinguishable from wild-type. (C and F) Deletion of Cx46 from MEK1(E) animals had no effect on eye and lens overgrowth. In contrast, MEK1(E)-Cx50KO and MEK1(E)-Cx50KI46 ocular tissues were significantly smaller than those from the MEK1(E) transgenic animals ($p < 0.05$, values are the mean $\pm$ SD, $n = 4$ –16 animals for each genotype).

Figure 8. Histological analyses of wild-type, MEK1(E), and MEK1(E)-Cx50KO lenses. Sections from P7 lenses were stained with hematoxylin and eosin. Wild-type eyes revealed normal ocular development with no lens pathologies in the central (A) and cortical (D) regions. MEK1(E) lenses were characterized by a pronounced loss of eosin staining in the nucleus (B) and the presence of vacuoles in the lens cortex (E). MEK1(E)-Cx50KO lenses exhibited a mild loss of eosinophilic staining in the core of the lens (C); however, vacuole formation was not detected in the cortex (F).

MEK1(E) or FGFR activation produced significant increases in junctional coupling mediated by Cx50, but not Cx46, in

Figure 9. Quantitation of lens glucose concentrations in three week old wild-type, MEK1(E), MEK1(E)-Cx50KO, and Cx50KO lenses. MEK1(E) lenses exhibited a 60% increase in glucose concentration compared with wild type. Deletion of Cx50 alone did not alter lens glucose concentration compared with wild type. MEK1(E)-Cx50KO lenses were significantly different from the MEK1(E) transgenic alone ($p < 0.05$, Student's *t* test) and restored lens glucose to wild-type levels.

paired *Xenopus* oocytes and primary lens epithelial cells. Deletion or replacement of Cx50 in transgenic mice alleviated a variety of lens pathologies induced by transgenic MEK1(E) expression, whereas knockout of Cx46 had no effect. These improvements included a delay in lens opacification, elimination of lens rupture, a reduction of fiber vacuolization in the equatorial region, abolition of macrophthalmia, restoration of glucose levels, and normalization of epithelial proliferation. These results suggest that MEK1 specifically modulates coupling mediated by Cx50, but not Cx46, and that gap junctional communication and MAPK signaling may act synergistically in the development of the internal lens circulation, and the maintenance of osmotic balance.

Although we have documented a significant increase in Cx50-mediated coupling induced by MAPK signaling, the mechanism responsible remains unclear. At present, we have no evidence that Cx50 is phosphorylated after MEK activation, although this remains an intriguing possibility. The coupling changes could have resulted from an increase in unitary conductance, or channel open probability. Alternatively, it may have been because of an augmented rate of channel assembly. All of these parameters can be influenced

Table 1. Summary of osmotic defects in the different mouse models

Mouse	Cx46 gene	Cx50 gene	MEK1(E)	Macrophthalmia	Lens rupture	Vacuole formation	Glucose accumulation
Wild-type	+ / +	+ / +	—	No	No	No	No
MEK1(E)	+ / +	+ / +	+	Yes	Yes	Yes	Yes
Cx46KO	— / —	+ / +	—	No	No	No	N.D.
Cx50KO	+ / +	— / —	—	No	No	No	No
Cx50KI46	+ / +	46/46 ^a	—	No	No	No	N.D.
Cx46KO-MEK1(E)	— / —	+ / +	+	Yes	Yes	Yes	N.D.
Cx50KO-MEK1(E)	+ / +	— / —	+	No	No	No	No
Cx50KI46-MEK1(E)	+	46/46	+	No	No	No	N.D.

N.D., not determined.

^a 46/46, knockin mice which lack endogenous Cx50 and have extra copies of Cx46 in its place (White, 2002).

by phosphorylation of connexin subunits (Moreno and Lau, 2007) and could potentially result from Cx50 being phosphorylated either directly by MAPKs or indirectly via other kinases activated by the MAPK pathway. Future investigation of these parameters may help explain why Cx50 junctional conductance was specifically increased compared with that mediated by Cx46.

We have identified reciprocal roles for MEK1 and Cx50 in osmotic regulation during postnatal fiber development and have summarized the rescue of osmotic defects caused by MEK1(E) by deletion or replacement of Cx50 in Table 1. Fiber gap junctional coupling contributes to an internal circulating current that underpins the nonvascular microcirculatory system used by the lens to maintain transparency (Mathias *et al.*, 1997; Donaldson *et al.*, 2001). Sodium is the primary ion carrying the circulating current, and it enters the lens at the anterior and posterior poles and flows inward along the extracellular spaces. Na⁺ is driven across fiber cell membranes by its electrochemical gradient, and in the fiber cytoplasm the direction of flow is reversed, with the current moving back toward the lens surface through gap junction channels (Mathias *et al.*, 2007). Because the coupling of differentiating fibers is normally highest at the equator and lowest at the poles (Baldo and Mathias, 1992), the intercellular current is directed to the equatorial epithelium where Na⁺/K⁺-ATPase activity is also concentrated (Gao *et al.*, 2000; Candia and Zamudio, 2002; Tamiya *et al.*, 2003), and sodium is pumped out of the lens to complete the circuit. Water and dissolved metabolites follow the Na⁺ current creating the microcirculatory system for the avascular lens.

Our data support a model in which the normal gradient in junctional communication is established by specific interaction between MAPK signaling and Cx50. The tremendous increase in lens mass resulting from the MEK1(E) transgene has been shown previously to be primarily a result of water accumulation within the lens (Gong *et al.*, 2001), implying an osmotic imbalance that could be driven by ion and solute accumulation within the lens. This notion is supported by the vacuole formation and glucose accumulation in MEK1(E) lenses that accompany the increase in water content. The highest levels of MAPK signaling are normally found at the lens equator (Le and Musil, 2001; Lovicu and McAvoy, 2005), and in mice expressing constitutively active MEK1(E) throughout the lens, this gradient is likely to be disrupted. The specific interaction between MEK1(E) and Cx50 could result in increased fiber coupling in other regions of the lens than the equator, disrupting the normal angular variation in fiber cell junctional coupling. Because the extensive vacuolization of fiber cells in the MEK1(E) lenses, it impossible to directly test this hypothesis by mea-

suring the angular variation in coupling by using whole-lens impedance techniques (Martinez-Wittingham *et al.*, 2003). However, this idea is supported by the elimination of glucose accumulation, fiber vacuolization, macrophthalmia, and lens rupture by the specific deletion of Cx50 from MEK1(E) transgenic mice.

Constitutive activation of a potent signal transduction pathway would be expected to wreak havoc with normal processes of development, as was found to be the case with MEK1(E) transgenic animals (Gong *et al.*, 2001). In recent years an unexpected role for connexins in lens development has emerged from numerous studies of genetically engineered mice (White *et al.*, 1998; Rong *et al.*, 2002; Sellitto *et al.*, 2004; Xia *et al.*, 2006). Although the deficiencies in cell proliferation and differentiation that result from genetic mutation of Cx50 are less severe than the developmental defects that arise from the experimental manipulation of MAPK signaling (Robinson *et al.*, 1995; Lovicu and McAvoy, 2001; Xie *et al.*, 2006; Zhao *et al.*, 2008), there is potential overlap in regulation of some aspects of development. Here, we have shown that simply removing Cx50 as one potential target for MAPK signaling can significantly diminish the developmental chaos induced by MEK1(E) expression in the lens. These data suggest that signal transduction and gap junctional communication may specifically interact in the maintenance of osmotic balance, and broaden the paradigm of how intercellular communication contributes to the regulation of development of specialized organs like the lens.

ACKNOWLEDGMENTS

This work was supported by National Eye Institute grants EY13163 (to T.W.W.) and EY13869 (to M. S.).

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