Nucleotide- and Protein-Dependent Functions of Actg1

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INTRODUCTION

Actin is an essential cellular protein involved in many important functions including cell motility, cytokinesis, muscle contraction, structural support, and regulation of gene expression (Pollard and Cooper, 2009). In mammals, these functions are carried out by the actin family of proteins, which is composed of the four muscle-specific actins, α-skeletal, α-smooth, α-cardiac, and γ-smooth, and the two ubiquitously expressed cytoplasmic actins, β- and γ-actin, each expressed from a unique gene. As a key component of many essential cellular processes, it is unsurprising that mutations in either cytoplasmic actin gene are associated with severe developmental defects in humans, and as β- and γ-actin are also important constituents of the stereocilia in the inner ear, mutations also often lead to different kinds of syndromic and nonsyndromic deafness (Zhu et al., 2003; Rivière et al., 2012; Rubenstein & Wen, 2014; Latham et al., 2018; Miyajima et al., 2020).

While all six mammalian actins are highly similar, the cytoplasmic actins share 99% sequence identity, differing by only 4 of 375 amino acids. The cytoplasmic actin gene sequences, Actb and Actg1, are highly conserved and also very similar, sharing 89% of their coding sequences in mice (Perrin and Ervasti, 2010). However, despite the high sequence similarity, the cytoplasmic actins seem to occupy unique biological niches, as demonstrated by the distinct differences in their respective gene knockout models. Actb−/− mice are embryonic lethal, with all embryos dying by E8.5 (Shawlot et al., 1998; Shmerling et al., 2005; Bunnell et al., 2011) while Actg1−/− mice are viable with increased perinatal lethality (Belyantseva et al., 2003; Miyajima et al., 2020).
The specific mechanisms by which nearly identical β- and γ-actin function differently remain an outstanding question. Current data suggest that functional differences between them may be due to isoform specific interactions with actin-associated proteins, distinct patterns of posttranslational modifications, or variations in mRNA abundance and localization (reviewed in Kashina, 2020). At the protein level, a number of studies have reported isoform-specific interactions with different actin-binding proteins. β-Actin has shown preferential binding to myosin 2B, tropomyosin (Pathan-Chhatbar et al., 2018), myosin 2C1 (Müller et al., 2013), betacap73 (Shuster et al., 1996; Welch et al., 2005), and DIAPH3 (Chen et al., 2017), while γ-actin shows preference for myosin 7a (Müller et al., 2013). Between the two cytoplasmic actins, studies have shown that only β-actin undergoes N-terminal arginylation (Karakozova et al., 2006; Kashina, 2006; Saha et al., 2010; Pavlyk et al., 2018); however, a new study suggests that N-terminal arginylation of β-actin is nominal in the presence of very high rates of N-terminal acetylation (Drazic et al., 2021). β- and γ-actin have also shown unique cellular distribution in different tissues and during different cell stages, with β-actin localizing to the cleavage furrow and γ-actin to the cell cortex during cell division (Otey et al., 1986, 1988; Dugina et al., 2009; Chen et al., 2017). The Actb mRNA contains a unique “zipcode” sequence in the 3’ UTR that interacts with RNA localization proteins, such as Zipcode binding protein 1, to promote local translation of the β-actin protein (Kislauskis et al., 1994; Ross et al., 1997; Hüttemaier et al., 2005; Pan et al., 2007).

While much has been revealed about the different roles of the two cytoplasmic actins, the essential differences conferring unique impacts on organismal survival have recently been linked to the nucleotide sequence of Actb, rather than the β-actin amino acid sequence. We and others (Vedula et al., 2017; Patrinostro et al., 2018) used different gene editing technologies to generate mice that expressed γ-actin protein from Actb, establishing a β-actin protein specific knockout that maintains an intact Actb nucleotide sequence, named Actb⁻γ⁻. In direct contrast to the embryonic lethality of the Actb⁻γ⁻ mouse (Bunnell et al., 2011), the Actb⁻γ⁻ mice were largely phenotypically normal with no defect in survival (Patrinostro et al., 2018; Vedula et al., 2017). Actb⁻γ⁻ MEFs also had normal proliferation rates and migration patterns (Patrinostro et al., 2018; Vedula et al., 2017). These results demonstrated that it is not the loss of β-actin that causes embryonic lethality in Actb⁻γ⁻ mice, but rather the loss of the intact Actb nucleotide sequence, suggesting that Actb must have protein-independent functions. However, the Actb⁻γ⁻ mice also developed progressive hearing loss due to degradation of inner ear hair cell stereocilia, supporting a tissue-specific function for β-actin protein (Patrinostro et al., 2018).

In this study, we assessed whether the Actgt1 nucleotide sequence also supports essential protein-independent functions by generating a novel mouse model where the only cytoplasmic actin expressed is γ-actin from Actb⁻γ⁻, named bG/0. We found that these mice are viable and express γ-actin protein levels no different from those in control littermates. However, we observed unique phenotypes that suggest that Actgt1 and γ-actin have nucleotide- and protein-dependent functions that differ from those of Actb or β-actin. Together, these results reveal novel insights into the differential functions of the highly similar cytoplasmic actins, and further support previous studies implicating the importance of nucleotide specific differences between Actb and Actgt1.

**RESULTS**

Mice expressing exclusively γ-actin from Actb are viable

To determine if reducing the cytoplasmic actin pool in mice to γ-actin expressed exclusively from the edited Actb gene supports viability, we crossed Actb⁻γ⁻ mice (Patrinostro et al., 2018) with Actgt1⁻⁻ mice (Belyantseva et al., 2009) to generate Actb⁻γ⁻ Actgt1⁻⁻ mice. Hereafter, Actb⁻γ⁻ Actgt1⁻⁻ will be referred to as bG/0, where the lowercase letter indicates the gene, the uppercase case letter indicates the protein expressed, 0 indicates Actgt1⁻⁻, and γ⁻ indicates Actgt1⁻⁻ (Supplemental Table S1). The bG/0 mouse were viable but were observed in sub-Mendelian ratios at weaning: 35.02% bG/gG, 58.23% bG/gG, and only 6.75% bG/0 rather than the expected ratios of 25:50:25. bG/0 mice presented with a median survival of only 163 d, which is significantly less than bG/gG or bG/gG littermates (Figure 1A). bG/0 mice were also significantly smaller than their bG/gG or bG/gG littermates, and male bG/0 mice were significantly smaller than WT controls (Figure 1, B and C). Decreased survival and size of the bG/0 mouse is consistent with results previously reported for Actgt1⁻⁻ mice (Belyantseva et al., 2009).

Because hypomorphic expression of cytoplasmic actins would explain the decreased viability in bG/0 mice most simply, we utilized quantitative real-time PCR (qRT-PCR) to measure isoacon transcript levels in WT, bG/gG, bG/gG, and bG/0 brain, lung, and MEF tissue. bG/0 lung and MEF total transcript levels were not significantly different from those in WT controls, while brain tissue showed a significantly increased level of total actin transcript in all bG/gG,
bG/gG/+ and bG/0 samples (Supplemental Figure S1). Upon loss of intact Actg1 in bG/0 mice, the tissues showed loss of expression of the Actg1 transcript and a corresponding increase in expression of the Actb–/– transcript. bG/0 lungs also showed a nonsignificant increase in Acta2 expression while MEFs showed a significant increase in Acta2 (Figure 2, A–F). These data suggest that the bG/0 tissues compensate for loss of Actg1 by up-regulating expression of other actin isoforms, including the edited Actb–/– allele.

We also measured relative protein levels of cytoplasmic, αsm-, and total actin using quantitative Western blotting of WT, bG/gG, bG/gG/+, and bG/0 brain, lung, and MEF tissue. Similar to the measured transcript levels, cytoplasmic actin protein levels appeared to undergo compensatory up-regulation to maintain a WT level of total actin protein. Despite the loss of Actg1, we measured γ-actin protein levels that remain constant across bG/gG, bG/gG/+, and bG/0 mice (Figure 3, A–F), demonstrating that the Actb–/– allele is able to compensate for loss of endogenous γ-actin expressed from Actg1.

During this transition, cytoplasmic actin polymerizes into stress fibers and cells up-regulate expression of αsm-actin (Tomasek et al., 2002; Hinz, 2007; Davis and Molkentin, 2014). We have previously observed myofibroblast-like phenotypes in Actb–/–, Actg1–/–, and Actb–/–/Actg1–/– MEFs (Patrinostro et al., 2017). Despite unchanged levels of αsm-actin (Figure 3C), we quantified stress fiber numbers and thickness in phalloidin-stained cells to determine if there might be other myofibroblast-like phenotypes in bG/0 MEFs.

In bG/0 MEFs, no change in fiber thickness or number was observed from those in WT and bG/gG controls (Figure 4C). MEFs have the capacity to differentiate into myofibroblasts in response to a number of environmental stimuli.

FIGURE 2: The Actb–/– transcript is upregulated with ablation of Actg1. (A–C) Absolute and (D–F) proportional quantification of isoactin and Actb–/– transcript in brain, lung, and MEFs of WT, bG/gG, bG/gG/+, and bG/0 mice and embryos (n = 4, in triplicate). Transcript amounts (picomoles) were calculated using a standard curve, amplified in parallel. For A–C, two-way ANOVA with Bonferroni posttest was performed. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Error bars are SEM. Comparisons are not statistically significant unless otherwise indicated.
Mice expressing γ-actin from Actb<sup>–<sup>g</sup> maintain WT levels of total actin protein. (A-C) Relative actin isoform protein expression in WT, bG/gG, bG/gG<sup>+/–</sup>, and bG/0 brain, lungs, and MEFs (n = 4). x-axis denotes actin isoform and y-axis denotes relative protein expression normalized to GAPDH and relative to WT. (D-F) Representative Western blots of brain, lung, and MEFs. Two-way ANOVA with Bonferroni posttest was performed. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Error bars are SEM. Comparisons are not statistically significant unless otherwise indicated.

G-actin for total actin from those in WT and bG/gG controls (Figure 4G). The significance of the G-actin decrease for total actin is likely due to the combined insignificant decrease observed for both γ- and α<sub>sm</sub>-actin.

One of the key signaling pathways that responds to changes in the polymerization state of actin is the serum response factor (SRF)/myocardin-related transcription factor (MRTF) signaling pathway (Vartiainen et al., 2007; Olson and Nordheim, 2010; Baarlink et al., 2013; Esnault et al., 2014). Quantitative Western blotting revealed no change in SRF or MRTF-A expression in bG/0 MEFs from WT, bG/gG, and bG/gG<sup>+/–</sup> (Figure 4H). Changes in cellular F-actin also impacts the Hippo signaling pathway through central effectors Yes-associated protein 1 (YAP) and transcriptional coactivator with PDZ binding motif (TAZ; reviewed in Seo and Kim, 2018). To gauge the impact of the bG/0 genotype on the Hippo pathway, we quantified YAP expression using quantitative Western blotting; no significant differences were observed (Figure 4H).

Finally, we quantified bG/0 MEF migration habits using a random cell migration assay (Figure 5, A–C). We observed that bG/0 MEFs migrated at rates similar to those in WT and bG/gG controls. No significant differences were observed for bG/0 directionality, direction autocorrelation, mean squared displacement (MSD), or speed from WT and bG/gG controls (Figure 5, D–G). These results lead us to conclude that the intact Actg1 nucleotide sequence is not required for cell migration.

**Loss of Actg1 results in a unique myopathy**

While β- and γ-actin are expressed in miniscule amounts in adult skeletal muscle compared with α-skeletal actin (Goldberg et al., 1980; Hanft et al., 2006), muscle-specific knockout of either Actb or Actg1 results in a mild, but progressive age-dependent myopathy (Sonnemann et al., 2006; Prins et al., 2011). However, conversion of β-actin to γ-actin protein via gene editing had no effect on muscle function (Patrinostro et al., 2018). bG/0 muscles were not different from WT or bG/gG controls in the percentage of centrally nucleated fibers (CNF), susceptibility to eccentric contraction induced force loss, muscle mass, fiber size, or fiber number (Figure 6, A and B; Supplemental Figure S3), but bG/0 muscles did present with significantly decreased specific isometric force (Figure 6C). bG/0 mice also displayed a hyperactivity phenotype in an open field assay (Supplemental Figure S4). Additionally, γ-actin protein expression in bG/0 muscle was not different from that in bG/gG (Figure 6D), suggesting that the myopathy is not due to hypomorphic γ-actin expression. Because these data suggest that bG/0 myopathy is not due to altered γ-actin levels, and because the myopathy differs from both Actb and Actg1 conditional muscle-specific knockout models, we conclude that the novel skeletal muscle weakness of bG/0 is due to the loss of Actg1 from a nonmuscle cell or tissue that supports skeletal muscle function, or loss of Actg1 in the earliest stages of development.

**Actg1 knockout does not exacerbate hearing phenotypes of Actb<sup>–</sup>g mice**

Based on studies in gene knockout and Actb<sup>–</sup>g mice, both cytoplasmic actins have been shown to be important for maintenance of stereocilia in the inner ear with loss of either causing progressive hearing loss (Perrin et al., 2010; Patrinostro et al., 2018). To determine if the bG/0 genotype further compromises the structure or function of the inner ear, we employed scanning electron microscopy (SEM) and auditory brainstem response (ABR) testing in 6 wk-old and 16 wk-old WT, bG/gG, and bG/0 mice. Outer hair cell (OHC) stereocilia in 6 wk-old bG/0 mice had normal morphology. In
contrast, at 16 wk of age we observed variable lengths in OHC stereocilia rows 2 and 3 from both bG/gG, and bG/0 mice, which resembles the Actb\(^{-/-}\), rather than the Actg1\(^{-/-}\) phenotype. Additionally, some OHCs were lost in the base of cochlea (Figure 7, A and B). The ABR thresholds of bG/0 were not significantly different from those of bG/gG at either 6 or 16 wk of age, and both lines showed significant hearing loss at high frequencies compared with WT mice at 16 wk of age (Figure 7, C–D). This pattern of progressive high-frequency hearing loss is again consistent with that previously seen in hair cell-specific Actb\(^{-/-}\) mice, but is different than that in Actg1\(^{-/-}\) mice, which had progressive hearing loss at all sound frequencies. These data suggest that the Actg1 nucleotide sequence is not necessary for auditory function because OHC structure and ABR thresholds were similar in bG/gG and bG/0 mice.

**DISCUSSION**

Previous data using various Actb\(^{-/-}\) models suggested that loss of \(\beta\)-actin is lethal in mice, leading to the conclusion that \(\beta\)-actin is an essential cellular protein (Shawlot et al., 1998; Shmerling et al., 2005; Bunnell et al., 2011). However, more recent studies utilizing CRISPR/Cas9 or TALENs generated a \(\beta\)-actin protein-specific knockout by editing the Actb nucleotide sequence to express \(\gamma\)-actin. These Actb\(^{-/-}\) mice were overtly normal, demonstrating that it is not the \(\beta\)-actin protein that is essential for mouse development, but rather the intact Actb nucleotide sequence, suggesting that Actb has protein-independent function (Vedula et al., 2017; Patrinostro et al., 2018). An attempt was made to generate a mouse that expressed \(\beta\)-actin from Actg1, but only three of the four amino acids were successfully edited. The partially edited Actg1 mouse presented with no abnormal phenotypes, suggesting that the survival defect observed in Actg1\(^{-/-}\) mice may also be due to loss of intact Actg1 nucleotide sequence (Vedula et al., 2017). Here we addressed whether expression of \(\gamma\)-actin exclusively from the edited Actb\(^{-/-}\) allele could support mouse and cell viability. Most interestingly, our data revealed that the bG/0 mouse present with significantly impaired survival, while expressing the same relative amount of \(\gamma\)-actin protein as the Actb\(^{-/-}\) line with normal survival. Our data support an important protein-independent role for the Actg1 nucleotide sequence.

Data collected here corroborate years of studies that emphasize that despite high sequence identity between \(\beta\)- and \(\gamma\)-actin, and their respective nucleotide sequences Actb and Actg1, the cytoplasmic actin genes and proteins have unique functions. Many of these studies have centered on genetically modified mice, and the

**FIGURE 4:** No growth or morphology phenotypes in bG/0 MEFs. (A) Representative images of WT, bG/gG, and bG/0 MEFs. \(\gamma\)-actin is labeled in magenta, \(\beta\)-actin is labeled in green, and nucleus is labeled in blue. Scale bar: 50 \(\mu\)m. (B) Representative images of phalloidin-stained actin filaments in WT, bG/gG, and bG/0 MEFs. Scale bar: 20 \(\mu\)m. (C) MEF growth curve of WT, bG/gG, bG/gG\(^{-/-}\), and bG/0 embryos cultured for 6 d (\(n=4\)). (D) Quantification of peak-valley ratios across linescans as a measure of stress fiber thickness and (E) relative number of fibers per cell (\(N=3, n=6\)). Each color represents an independent experiment. (F) Representative linescans of WT, bG/gG, and bG/0 MEFs. (G) Ratio of G- to F-actin for \(\beta\)-, \(\gamma\)-, \(\alpha\)\(_{sm}\)-, and total actin in WT, bG/gG, and bG/0 MEFs (\(n=3\)). (H) Relative expression of actin-associated proteins SRF, MRTF-A, and YAP normalized to GAPDH in WT, bG/gG, bG/gG\(^{-/-}\), and bG/0 MEFs (\(n=4\)). One- or two-way ANOVA with Bonferroni posttest was performed. For D and E, statistical analysis was performed on the means of independent experiments. Error bars are SEM. *\(p<0.05\), **\(p<0.01\), ***\(p<0.001\), ****\(p<0.0001\). Comparisons are not statistically significant unless otherwise indicated.
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results of these various mouse models reveal that we cannot predict if an organismal function requires the cytoplasmic actin nucleotide sequence or protein, despite the high similarity of the two. While mouse survival is dependent on Actb (Bunnell et al., 2011), we confirm here that it is also negatively impacted by loss of Actg1. Previous studies attributed the importance of γ-actin or Actg1 for mouse lifespan and body mass to the protein (Belyantseva et al., 2009; Bunnell & Ervasti, 2010), but here we demonstrate that the Actg1 nucleotide sequence is required for normal mouse survival and growth. On the other hand, we have also identified protein-dependent functions for γ-actin that are not the same for β-actin. The Actb nucleotide sequence is required for cell proliferation (Bunnell & Ervasti, 2010; Patrinostro et al., 2017), but here we showed that it is the loss of γ-actin protein that impairs proliferation in Actg1−/− MEFs rescued by reintroduction of γ-actin in the bG/0 mice (summarized in Table 1).

The phenotypic consequences of perturbing either a cytoplasmic actin gene or a protein were also observed through a novel skeletal muscle weakness phenotype in bG/0 mice. bG/0 muscle weakness was characterized by a significant decrease in specific iso-

FIGURE 5: Normal cell motility in bG/0 MEFs. (A-C) Individual cell trajectories mapped from the origin for WT, bG/gG, and bG/0 MEFs for each genotype to characterize random migration patterns. Each different colored line indicates an individual cell path. (D) Directionality, (E) directional autocorrelation, (F) mean square displacement, and (G) speed; N = 4, n = 10 cells per embryo. For G, each color represents an independent experiment. For D-F, two-way ANOVA with repeated measures and Bonferroni posttest was performed. For G, one-way ANOVA with Bonferroni posttest was performed; statistical analysis was performed on the means of independent experiments. Error bars are SEM. Comparisons are not statistically significant unless otherwise indicated.

strength. Alternatively, this discrepancy between models may arise from small differences in the knockout mechanism. The Actg1−/− knockout in bG/0 is constitutive and present in the earliest stages of embryogenesis (Belyantseva et al., 2009), while muscle-specific Actg1−/− is a conditional knockout that is triggered by expression of Cre from the human α-skeletal actin (HAS) promoter, which only begins to express around 9 d post coitum (dpc; Miniou et al., 1999). Therefore, if intact Actg1 is required in the earliest stages of muscle development, the muscle-specific Actg1−/− mice may escape the myopathy observed in the bG/0 mice.

Exactly how the nucleotide sequences of the cytoplasmic actin genes confer their important functions remains elusive. Multiple lines of evidence identify functional noncoding regions of either gene. The importance of Actb may be due to local translation of the transcript via the Actb zincode (Kislauskis et al., 1994; Ross et al., 1997; Artman et al., 2014). Other regulatory elements have been identified in the Actb 3′ UTR and intron 3 of Actg1 (DePonti-Zilli, Seiler-Tuyns, & Paterson, 1988; Lloyd & Gunning, 1993; Drummond & Friderici, 2013), suggesting these or other unidentified functional regions within noncoding sequences of either gene may serve Actb or Actg1 nucleotide-dependent functions. While the aforementioned regions are largely involved in regulating expression of β- and γ-actin, other sequence elements could be involved in various cell functions through regulating expression of other genes. Microarray analysis of Actb−/− MEFs revealed dysregulation of genes involved in the cell cycle, actin dynamics, and myosin activity (Bunnell et al., 2011); however, further characterization studies are needed to identify if Actg1−/− cells have similar expression changes and to clarify how either gene may be causing changes in gene expression.
In addition to demonstrating nucleotide-dependent functions for Actg1 in mouse survival and skeletal muscle strength, we have also confirmed protein-dependent functions of cytoplasmic actins in hearing function and stereocilia structure in the inner ear. γ-Actin-specific functions might be revealed if the fully edited Actg1<sup>c–b</sup> mouse were generated. Vedula et al. (2017) attempted to generate this γ-actin-specific knockout, but was only partially successful, so another attempt to complete this model would be beneficial in fully defining the differential roles of the cytoplasmic actin proteins. However, from a completely different perspective, we examined this same question with the bG/0 mice to uncover novel data identifying protein-independent functions of Actg1. Further investigation into the protein-independent functions of Actb and Actg1 will be essential to determining how these nucleotide sequences are conferring important functions within an organism and likely reveal novel roles for noncoding DNA that may be relevant to other genes. Cytoplasmic β- and γ-actin have been highly evolutionarily conserved from birds to mammals, despite being 99% similar at the amino acid level and 89% identical at the nucleotide level (Perrin and Ervasti, 2010). Further clarification on the functional differences between the two will provide novel insights into the evolutionary significance of highly similar molecules that might be applied to other proteins and/or genes.

Another theory posits that differential translation rates resulting from the higher percentage of noncoding nucleotide differences between Actb and Actg1 contribute to the importance of the cytoplasmic actin genes. It has been observed that γ-actin from Actg1 is translated more slowly than β-actin from Actb, and these differential translation rates confer differences in focal adhesion turnover and cell migration (Zhang et al., 2010; Vedula et al., 2021).

Recent work by Vedula et al. (2021) found that exogenous expression of the γ-actin coding sequence flanked by the Actb promoter and 3' untranslated sequence resulted in increased directional migration rates in immortalized MEFs, but had no effect on random cell migration rates, while expression of the β-actin coding sequence in the same context decreased migration rates. In this study, we found that exclusively expressing endogenous Actb-coded γ-actin in the bG/0 mice had no significant impact on random cell migration rates. We did not assess directional migration in bG/0 MEFs because previous work on Actg1<sup>c–b</sup> MEFs found that directional migration was unaffected by loss of the Actg1 coding sequence and γ-actin protein (Bunnell and Ervasti, 2010). Disparities between these studies suggest that differences in the biological system may alter cytoplasmic actin function. Migration studies in Actg1<sup>c–b</sup> and bG/0 cells were conducted in primary cells with germ-line gene edits, while the study by Vedula et al. (2021) was conducted in immortalized cells expressing both exogenous and endogenous cytoplasmic actins.

**MATERIALS AND METHODS**

Request a protocol through Bio-protocol.

**Mice**

Animals were housed and treated in accordance with the standards set by the University of Minnesota Institutional Animal Care and Use Committee. All animal experiments were approved by the University of Minnesota Institutional Animal Care and Use Committee under protocol numbers 1806A36018 and 2106A39169. Mice were housed in a specific pathogen-free facility on a 12 h light/dark cycle with ad libitum access to food and water. All mice used in this study were on the C57BL/6J background. Actb<sup>c–g</sup>−<sup>g</sup>− (Patrino et al., 2018) and Actg1<sup>c–g</sup>−<sup>c–g</sup>− (Belyantseva et al., 2009) mice were crossed and genotypes were determined as described previously (Patrino et al., 2018; Sonnemann et al., 2006). Mice were killed by cervical dislocation after anesthesia with Avertin at 3 mo of age for phenotypic analysis. Tissue was dissected and snap-frozen in liquid nitrogen.

**Cell Culture**

Primary WT, bG/gG, bG/gG<sup>c–g</sup>−, and bG/0 mouse embryonic fibroblasts (MEFs) were isolated from E13.5 embryos as described previously (Bunnell and Ervasti, 2010). MEFs were grown in approximately 80% confluency in MEF media (DMEM supplemented with 10% fetal bovine serum, 1% Pen/Strep, and 0.5 μg/mL Fungizone) and 1 x 10<sup>6</sup> cells were frozen at passage one in MEF freezing media.
(95% fetal bovine serum + 5% DMSO). MEFs were then thawed and cultured in MEF media.

**Mouse embryonic fibroblast proliferation assay**
MEFs were seeded at a density of $5 \times 10^4$/well of a six-well plate, in duplicate, in MEF media and a single well of each plate was counted every day for 6 d using a hemocytometer.

**Mouse embryonic fibroblast fixation, staining, and immunofluorescent imaging**
Coverslips were coated in 5 µg/ml fibronectin, seeded with $2 \times 10^4$ MEFs, and incubated overnight in MEF media. The following day, cells were fixed with 4% paraformaldehyde in PBS for 30 min at room temperature (RT), washed 3 x 5 min with phosphate-buffered saline (PBS), permeabilized for 10 min at RT with 0.1% Triton in PBS, and blocked for 30 min at RT with 3% bovine serum albumin (BSA) + 0.1% Triton in PBS. Coverslips were washed once with 0.1% Triton in PBS. For isoform-specific staining, coverslips were stained with the following primary antibodies in 3% BSA + 0.1% Triton in PBS overnight at 4°C: β-actin (1:400; Sigma-Aldrich, AC15) and γ-actin (affinity-purified γ-cyto actin rabbit 7577). For F-actin staining, coverslips were stained with Acti-Stain 555 phalloidin (cytoskeleton PHDH1-A) according to manufacturer’s instructions. All coverslips were then washed 3 x 5min with PBS, rinsed with ddH$_2$O, and mounted with ProLong Gold Antifade with 4′,6-diamidino-2-phenylindole (DAPI) (Cell Signaling Technology 8961S). Images were collected using a 20×/NA0.75 or 60×/NA1.42 objective on a DeltaVision personalDV microscope with softWorx 7.2.1 (GE Technologies) using the same laser intensities and exposure times.

**Image analysis**
Images were analyzed using ImageJ (National Institutes of Health, Bethesda, MD, version 1.52p). Actin stress fiber thickness was quantified using fluorescence intensity of linescans across the widest portion of the cell body perpendicular to the fibers. The peaks and corresponding valleys were then determined, excluding the first and last peaks, which correspond to the edges of the cell. Fiber number totals were measured as the total number of peaks normalized to the width of the linescan. To quantify the proportion of peripheral

![FIGURE 7: bG/0 mice have progressive hearing loss. (A, B) SEM images and (C, D) ABR of 6 wk- and 16 wk-old WT, bG/gG, and bG/0 mice (n ≥ 3). SEM images of cochlea middle turn OHC. Scale bar: 1 µm. ABR defined frequency in kilohertz is on the x-axis and threshold (in decibels) sound that elicits a response is on the y-axis. One-way ANOVA with Bonferroni posttest was performed. *P < 0.05, **P < 0.01, ***P < 0.001. Error bars are SD. For significance in D, Top symbols: bG/0 compared with WT; Bottom symbols: bG/gG compared with WT. Comparisons are not statistically significant unless indicated otherwise.

| Function                        | Actg1 | γ-actin |
|---------------------------------|-------|---------|
| Mouse survival                  | +     | -       |
| Mouse growth                    | +     | -       |
| MEF proliferation               | -     | +       |
| Skeletal muscle strength        | +     | -       |
| Hearing                         | -     | +       |
| OHC stereocilia maintenance     | -     | +       |

Note: A “+” indicates that Actg1 or γ-actin is involved in the corresponding function while a “-” indicates it is not involved.

**TABLE 1: Functions dependent on the Actg1 nucleotide sequence and γ-actin protein.**
γ-actin, cells were masked and all background fluorescence was cleared. Cell masks were then outlined and dilated to define the cell periphery. From these measurements, the ratio of raw fluorescence intensity in the cell periphery to the raw fluorescence intensity of the internal area was then calculated. The cell masks were also used to measure circularity and aspect ratio.

Live cell imaging
A sample of 2 × 10⁶ MEFs were cultured overnight in MEF media in a Nunc glass-bottomed dish (Thermo Scientific 150680). The following day, the MEF media was replaced with MEF media without phenol red + HEPES to stabilize the pH and the dishes were sealed with vacuum grease and a glass coverslip. Images were collected every 10 min for 4 h using a 10x/NA0.25 objective with phase contrast illumination on a DeltaVision personalDV in an environmental chamber maintained at 37°C. Cells were tracked using the Manual Tracking plugin for ImageJ (National Institutes of Health, Bethesda, MD, version 1.52 h) and the xy track data was analyzed using the DiPer plugin for Excel (Gorelik and Gautreau, 2014). Cells that divided or contacted other cells were excluded from analysis.

qRT-PCR
WT and Actb−/− mouse isoactin controls were generated and respective primer sets were verified previously to be isoform-specific (Patrinostro et al., 2017, 2018). For whole tissue, samples were pulverized using liquid nitrogen and a mortar and pestle and homogenized in Trizol using a 27G needle and syringe. Total RNA was extracted from homogenized tissue and MEF samples using the Bio-Rad Aurum Total RNA Mini-Kit (7326820) according to manufacturer’s instructions. RNA concentration was measured using a NanoDrop 1000 spectrophotometer (ThermoFisher Scientific). A Bio-Rad iScript Advanced cDNA synthesis kit was used to synthesize first-strand cDNA from a standard amount of RNA. Isoactin and Actb−/− control samples were used in a series of 10-fold dilutions to generate standard curves, and MEF or tissue cDNA samples were amplified in parallel using Bio-Rad SsoAdvanced Universal SYBR Green Supermix (1725270) and isoform-specific primers using the Bio-Rad CFX96 Real Time System C1000 touch thermal cycler. Transcript quantities were calculated in picomoles using the standard curves.

Western blotting
Brain, lungs, and gastrocnemius muscles were pulverized in liquid nitrogen using a mortar and pestle. MEF, brain, and lung protein was extracted in 1% SDS buffer in 1X PBS with a cocktail of protease inhibitors (100 µM aprotonin, 0.79 mg/ml benzamidine, 1 µM calpain, 1 µM calpeptin, 10 µM E-64, 10 µM leupeptin, 0.1 µg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride) and MEF lysates were sonicated (Model 150V/T ultrasonic homogenizer; BioLogics). All samples were boiled and centrifuged to remove the insoluble fraction. Pulverized gastrocnemius muscle was subjected to low-salt extraction and DNase enrichment as described previously (Hanft et al., 2006). Equal amounts of cleared lysates (25 µg brain or lung, 20 µg gastrocnemius, and 15 µg MEF) were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) before being transferred to a PVDF membrane and blocked for 30 min in 5% nonfat milk in PBS. The following antibodies were used: β-actin (1:5,000; Sigma-Aldrich, AC15), γ-actin (1:5,000 mAB 2-4), α-sm-actin (1:5,000; Sigma-Aldrich, 1A4), Pan-actin (1:5,000; Seven Hills Bioreagents, C4), SRF (1:1,000; Santa Cruz Biotechnology, G-20), MRTF-A (1:1,000; Cell Signaling Technology, E2V21), or YAP (1:500, Abnova) with glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:5,000; Sigma-Aldrich, G9545) as a loading control and secondary antibodies DyLight 800 anti-mouse IgG (1:10,000; Cell Signaling Technology, 5257S) and DyLight 680 anti-rabbit IgG (1:10,000; Cell Signaling Technology, 5366S). Blots were imaged using the Odyssey CLx infrared scanner (LI-COR Biosciences) and protein bands were quantified using LI-COR Image Studio Software.

G- to F-actin ratio
Equal numbers of WT, bG/gG, and bG/0 MEFs were pelleted before the experiment. G- and F-actin fractions were isolated from MEFs using the G-actin/F-actin in vivo assay kit (Cytoskeleton, #BK037) according to manufacturer’s instructions and Western blotted for β-actin, γ-actin, α-sm-actin, and total actin (see Western Blotting for a complete list of antibodies). Blots were imaged using the Odyssey CLx Infrared scanner (LI-COR Biosciences) and fluorescence intensities of G- and F-actin protein bands were quantified using LI-COR Image Studio Software to determine the ratio of G- to F-actin for each genotype.

Open field activity assay
Mice were placed in an open-field apparatus for 15 min and total horizontal distance and vertical movement counts were measured based on infrared beam breaks. Activity was measured using the AccuScan system (Columbus Instruments).

Muscle immunofluorescence imaging
Quadiceps muscles from each mouse line were cryopreserved in melting isopentane for 30 s and 7-µm transverse cryosections were obtained (Leica CM3050 S). For immunofluorescence, sections were fixed in acetone at -20°C for 15 min and subsequently washed three times in PBS before being blocked in 5% goat serum for 30 min at RT. Sections were incubated for >1 h in primary antibody (rat monoclonal anti-Laminin 1:500; Sigma, L06631) at RT. Slides were then washed three times in PBS before incubation with Alexa Fluor 488 goat anti-rat IgG (1:1000; ThermoFisher, A-11006) secondary for 30 min at RT. Sections were finally washed three times in PBS and mounted in ProLong Gold Antifade with 4’,6- diamidino-2-phenylindole (DAPI) to visualize nuclei (ThermoFisher Scientific). Images were acquired on a Leica DM5500 B microscope equipped with a Leica HC PLAN APO 10x objective and stitched together with LASX software (Leica) to allow visualization of the entire quadriceps. SMASH—semi-automatic muscle analysis using segmentation of histology software—was used to analyze and quantify centrally located nuclei, fiber number, and fiber size (Smith and Barton, 2014).

Ex vivo EDL force measurements
Contractile function of EDL muscles was assessed according to methods described previously (Moran et al., 2005). Mice were anaesthetized with sodium pentobarbital (75-100 mg/kg body mass). EDL muscles were dissected and mounted on a 300B-LR dual-mode muscle lever system (Aurora Scientific) with 5-0 suture in a 1.2-ml bath assembly with oxygenated (95:5% O₂/CO₂) Krebs Ringer bicarbonate (Krebs) buffer maintained at 25°C. The stimulator and muscle lever system were controlled by computer using a KPCI-3108 interface board (Keithley Instruments) and TestPoint software (SuperLogics). Muscles were adjusted to their anatomical optimal length (Lₒ) based on resting tension, with length being measured from the distal myotendonous junction to the proximal myotendonous junction using digital calipers. Before eccentric contractions were performed, maximal isometric tetanic force (Pₒ) was measured every 2 min by stimulating the muscle to contract for 200 ms at 175...
Hz until force plateaued. A series of 10 eccentric contractions (ECC) were performed and the peak force of each contraction was recorded. For each ECC force measurement, the muscle was passively shortened to 95% L0 and then stimulated for 200 ms while the muscle was simultaneously lengthened to 105% L0 at a velocity of 0.5 L0/s. Each eccentric contraction was separated from the next eccentric contraction by 3 min of rest to prevent fatigue (Lowe et al., 1994). The force measured at each eccentric contraction was expressed as a percentage of the force produced during the first contraction.

**Auditory brainstem response**

Auditory brainstem response (ABR) waveforms for mice were collected using a Tucker Davis Technologies System 3 at frequencies of 4, 8, 11, 16, 22, and 32 kHz as described previously (Patrinostro et al., 2018). Scalp potentials were recorded using subdermal electrodes following anesthetization with Avertin. Waveforms for each frequency were collected starting at 90 dB, decreasing in 5-dB steps to a subthreshold level. The collected waveforms were stacked and averaged as a percentage of the force produced during the first contraction.

**Scanning electron microscopy**

Dissected cochlea were fixed in 2.5% glutaraldehyde, 0.1 M sodium cacodylate, 2 mM CaCl2 overnight at 4°C and then decalcified in 170 mM EDTA in PBS for 16 h at 4°C. Dissected organ of Corti was incubated in 2% each of arginine, glutamine, glycine, and sucrose in water overnight at RT, followed by incubation in 2% tannic acid and guanidine hydrochloride for 2 h at RT and 1% OsO4 in water for 1 h at RT, with extensive washes between steps. The samples were then treated to 100% ethanol, critical point dried from CO2 and sputter coating with gold. Samples were imaged using a JEOL JSM-7800F field emission scanning electron microscope.

**Statistics**

All statistics were calculated using GraphPad Prism Software (version 9.0.2). One- or two-way ANOVAs with Bonferroni posttest were performed based on the specific data set and significance was determined with *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

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