Corrections

NEUROBIOLOGY
Correction for “High-resolution structure of hair-cell tip links,” by Bechara Kachar, Marianne Parakkal, Mauricio Kurc, Yi-dong Zhao, and Peter G. Gillespie, which appeared in issue 24, November 21, 2000, of Proc Natl Acad Sci USA (97:13336–13341; 10.1073/pnas.97.24.13336).

The authors note that Figure 3 appeared incorrectly. The corrected figure and its legend appear below. This error does not affect the conclusions of the article.

Fig. 3. Upper and lower attachments of the tip link. (A and B) Freeze-etch images of tip-link upper insertions in guinea pig cochlea (A) and (left to right) two from guinea pig cochlea, two from bullfrog sacculus, and two from guinea pig utriculus (B). Each example shows pronounced branching. (C and D) Freeze-etch images of the tip-link lower insertion in stereocilia from bullfrog sacculus (C) and guinea pig utriculus (D); multiple strands (arrows) arise from the stereociliary tip. (E) Freeze-fracture image of stereociliary tips from bullfrog sacculus; indentations at tips are indicated by arrows. (Scale bars: A = 100 nm, B = 25 nm; C–E = 100 nm.)
BIOCHEMISTRY
Correction for “X-ray structure of the arenavirus glycoprotein GP2 in its postfusion hairpin conformation,” by Sébastien Igonet, Marie-Christine Vaney, Clemens Vonhrein, Gérard Bricogne, Enrico A. Stura, Hans Hengartner, Bruno Eschli, and Félix A. Rey, which appeared in issue 50, December 13, 2011, of Proc Natl Acad Sci USA (108:19967–19972; first published November 28, 2011; 10.1073/pnas.1108910108).

The authors note that, due to a printer’s error, the author name Clemens Vonhrein should instead appear as Clemens Vonrhein. The corrected author line appears below. The online version has been corrected.

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High-resolution structure of hair-cell tip links

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Transduction-channel gating by hair cells apparently requires a gating spring, an elastic element that transmits force to the channels. To determine whether the gating spring is the tip link, a filament interconnecting two stereocilia along the axis of mechanical sensitivity, we examined the tip link’s structure at high resolution by using rapid-freeze, deep-etch electron microscopy. We found that the tip link is a right-handed, coiled double filament that usually forks into two branches before contacting a taller stereocilium; at the other end, several short filaments extend to the tip link from the shorter stereocilium. The structure of the tip link suggests that it is either a helical polymer or a braided pair of filamentous macromolecules and is thus likely to be relatively stiff and inextensible. Such behavior is incompatible with the measured elasticity of the gating spring, suggesting that the gating spring instead lies in series with the helical segment of the tip link.

By opening or closing transduction channels, deflection of the mechanically sensitive hair bundle initiates mechanoelectrical transduction in hair cells (1). Bundle deflection stretches elastic elements, the gating springs, which transmit force to the hundred or so transduction channels per cell (2). The best candidate for the gating spring is the tip link, an extracellular filament that joins a stereocilium to its tallest neighbor, parallel to the bundle’s axis of mechanical sensitivity (3). Tip links should be stressed during excitatory stimuli, be slackened during inhibitory ones, and be unaffected by stimuli perpendicular to the sensitivity axis. When tip links were eliminated by brief exposure to the Ca²⁺ chelator 1,2-bis(O-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA), mechanoelectrical transduction disappeared and the bundle moved >100 nm, as if the gating springs had been abruptly disengaged (4). In addition, tip links regenerated within 24 h after BAPTA treatment, with mechanoelectrical transduction returning over the same time span (5). Because BAPTA rapidly severs tip links and has few effects on other stereociliary linkages, tip links either are the gating springs or are connected in series with them (4).

The molecular identity of the tip link is unknown. By transmission electron microscopy (TEM), tip links appear as amorphous filaments of ~5 nm diameter and 150–300 nm length (3, 6). The lower end of a tip link contacts an intracellular osmiophilic structure, the tip density, whereas the other end contacts another osmiophilic structure, the insertional plaque (6). Its sensitivity to proteases (7, 8) and ability to be labeled by polycationic compounds (9) suggests that the tip link is a glycosylated protein. If the tip link is the gating spring, biophysical estimates of its stiffness (~1 mN m⁻¹) indicate that it should withstand forces of >100 pN and stretch more than twice its resting length (2, 10). To further explore the function and molecular identity of the tip link, we have chosen to characterize its structural properties and its sensitivity to chemical perturbations.

To produce higher-resolution images of the tip-link fine structure, we used the rapid-freeze, deep-etch technique (11, 12), with improved metal replication and imaging procedures. We find that although it is likely to be an essential element for transduction, the tip link’s structure seems incompatible with previous suggestions that it makes up the entire elastic gating spring.
chickens (5). Papillae were incubated at room temperature in a standard saline solution (155 mM NaCl, 6 mM KCl, 3 mM D-glucose, 5 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid at pH 7.4), with or without added Ca\(^{2+}\). When measured by atomic absorption, our nominally Ca\(^{2+}\)-free saline contained 1.4 µM Ca\(^{2+}\). For measurement of pH sensitivity, the standard saline solution was buffered with 5 mM each of glycylglycine, acetic acid, 2-[N-morpholino]ethanesulfonic acid, N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid, and 3-[cyclohexylamino]-1-propanesulfonic acid. After treatment of basilar papillae, the pH of the solution was measured to confirm that the pH remained unchanged. After exposure to agents for 10 min, papillae were washed with saline solution, treated with 50 µg/ml subtilisin (Sigma protease type XXIV) to remove the tectorial membrane, fixed, and processed for scanning electron microscopy (SEM) as described (5). Proteases and chloride salts of divalent and trivalent cations were obtained from Sigma; glycosidases were purchased from Boehringer Mannheim. In some cases, papillae were cultured after BAPTA treatment as described (5); tip-link regeneration was qualitatively similar to that seen previously.

**Results**

**Resolution of Freeze-Fracture and Freeze-Etching Replicas.** Deposition of evaporated metal, required for imaging, greatly influences the appearance and resolution of freeze-fracture replicas (14). Several factors must be considered when using rotary-shadowed metal replicas of freeze-etched samples to characterize molecular structures by appearance. To produce a metal replica, high-energy atomic particles are used to bombard the tissue surface. During impact, the metal particles transfer some of their kinetic energy to delicate biological macromolecules; bombarding metal atoms embed into the surface, modifying surface structures and heating the specimen. Heating can rapidly degrade the low-mass, free-standing structure of the tip link. Continuous bombardment also produces nucleation and growth of small microcrystals (metal grains). In our freeze-etch replicas, initial bursts of high-energy platinum atoms evaporated from the electron beam gun are likely to lodge deep within freeze-dried tip-link structures. A low amount of bombarded metal atoms produces, at the expense of contrast and reproducibility, a finer positively stained image of smaller structure details. By contrast, when larger amounts of platinum are deposited, structures are covered with a frosting of microcrystallized platinum, which highlights other surface details (12). Metal grain sizes for a thin coat of platinum have diameters of 1–2 nm (14).

The appearance of tip links depended greatly on metal-evaporation parameters. Rotary evaporation (shadowing) produces optimal information of surface topography of objects, as long as they are symmetrical and properly positioned in relation to the evaporation angle. Despite controlling the thickness of the metal coating, the thickness of shadowed replicas varied significantly from place to place because of patterns and angles formed by the highly convoluted surfaces of hair bundles. Because they could be shadowed by stereocilia, tip links were often poorly exposed to the platinum rotary evaporation.

Replicas also can be eroded during tissue digestion and replica cleaning; in addition, resolution can be degraded by recrystallization of metal atoms during exposure of the replica to the imaging electron beam. Another significant limitation in freeze-etch imaging of hair bundles is that the resulting images are two-dimensional projections of three-dimensional structures; distance is often impossible to measure. Our situation differs from freeze-etch imaging on a mica surface, where the biological structure is entirely laid out on the two-dimensional surface (12).

Because of the relative scarcity of tip links and the technical difficulties mentioned above, our rate of successful imaging was very low. We made more than 120 replicas from about 40 guinea pigs and 40 bullfrogs. We photographed 58 tip-link images that we judged to be free of obvious specimen-preparation artifacts; about two-thirds of these were from the bullfrog sacculeus and the remainder were from guinea pig cochlea and utriculus.

**The Helical Structure of the Tip Link.** The best images of tip links were obtained from replicas produced with the smallest amounts of evaporated platinum and the highest amounts of carbon; our interpretation of key features is shown in Fig. 1A. Fig. 1B shows our best image of the extended portion of a tip link. This image shows a symmetrical shadow and very small grain sizes. Zero-loss imaging allowed considerable contrast enhancement despite the low amount of electron-dense platinum. The very small platinum grains appear to be embedded in the structure, giving the appearance of a positive contrast rather than a frosting coat. Because of the increased contrast generated with the zero-loss imaging, the images of the carbon coat are also enhanced and

![fig. 1](image1.jpg)

Fig. 1. Helical structure of the tip link. (A) Proposed model for tip-link structure. Two helically intertwined protofilaments (inset) make up the tip link, attaching at two points to the taller stereocilium and contacting three filaments emanating from the shorter stereocilium. (B) Freeze-etch image of the extended portion of a tip link. (C) Freeze-etch image of the extended portion of a tip link. (D) Surface plot of the pixel intensities of the digitized image of the tip link shown in B created with National Institutes of Health IMAGE. The pseudo-three-dimensional image helped visualize the helical configuration and the possible periodic structure of the protofilaments. (Scale bars: B = 50 nm; C and D = 10 nm.)
appear as very visible halos around the contoured surfaces, partially blurring the imaging of the platinum. A particularly heavy carbon coat was used in this replica, which protected the tip link’s platinum coat during tissue digestion and prevented radiation damage and platinum recrystallization during imaging.

A magnified image (Fig. 1C) shows clearly that the extended domain of the tip link is formed by a coiled double filament. The two protofilaments formed a right-handed helix with a 60-nm period. Although we noted a possible periodic substructure within each protofilament, more apparent in a surface plot of the magnified image (Fig. 1D), the periodicity was close to the limit of resolution using this technique.

Because irradiation damage and platinum recrystallization damaged and distorted tip links during imaging, we chose to emphasize images least damaged by the visualization technique. Consequently, tip links varied in appearance and diameter. Fig. 2A–C shows the same tip link viewed at different tilt angles, illustrating the changes in the thickness of the platinum image and the carbon halo from different angles, as well as demonstrating deterioration of the platinum image during continued irradiation. Fig. 2D shows a gallery of tip-link extended segments. Although the platinum grains are not as small as those in Fig. 1, these tip links also showed a 30-nm periodicity of thinner and thicker regions, consistent with the helical structure with 60-nm pitch seen clearly in Fig. 1. This periodicity was also impressed into the carbon halo. We made measurements of the diameter of the projected helical images of the tip links at different portions of the thinner and the thicker regions. The measured average diameter was 8.3 ± 0.2 nm (n = 10) at the thinner regions and 10.3 ± 0.3 nm (n = 23) at the thicker regions.

**Multiple Attachments of the Tip Link.** Attachment of the tip link to the stereocilia was different at each end. At the upper end, the tip link consistently split apart about 10–50 nm from the taller stereocilium, so that two branches attached to this stereocilium, and from bullfrog sacculus (three panels to the right). (Scale bars = 15 nm.)

**Fig. 2.** Variability in appearance of tip-link extended structure. (A–C) Images of the tip link of Fig. 1B acquired with different tilt angles showing different apparent thickness of the tip link and its carbon coat. Despite degradation of the platinum image of the tip link, the enveloping carbon coat still shows the 30-nm periodic widening (arrows) and narrowing consistent with the projected view of a helical structure. (D) Images of the extended structure of different tip links from guinea pig cochlea (first two panels from left to right) and from bullfrog sacculus (three panels to the right). (Scale bars = 15 nm.)

**Fig. 3.** Upper and lower attachments of the tip link. (A and B) Freeze-etch images of tip-link upper insertions in guinea pig cochlea (A) and (left to right) two from guinea pig cochlea, two from bullfrog sacculus, and two from guinea pig utriculus (B). Each example shows pronounced branching. (C and D) Freeze-etch images of the tip-link lower insertion in stereocilia from bullfrog sacculus (C) and guinea pig utriculus (D); multiple strands (arrows) arise from the stereociliary tip. (E) Freeze-fracture image of stereociliary tips from bullfrog sacculus; indentations at tips are indicated by arrows. (Scale bars: A = 100 nm, B = 25 nm; C–E = 100 nm.)

not apparent, and the remainder were inappropriately oriented for us to distinguish bifurcation. Although the branches appear to have a diameter about half that of the tip link (Fig. 3B), their fragility during irradiation made this estimation of very limited quantitative value. In some cases, we saw images where one of the branches appeared to be recoiled or retracted (Fig. 3B, first panel). Although the appearance of two branches at the point of insertion with the taller stereocilium has been previously observed by SEM (6) and tip-link bifurcation can also be occasionally observed in thin sections (Fig. 4D), these images show that branching is a consistent feature of tip-link structure.

The structure of the tip link’s insertion into the membrane of the shorter stereocilium was more difficult to resolve. Because the tip link inserts at a near-perpendicular angle, a side view of this insertion point is partially blurred by the carbon film that coats the membrane. In rare occasions where we could discern features of this insertion point, the tip link appeared to branch into or contact two or three strands, each ∼25–40 nm in length, which we call anchor filaments (Fig. 3C and D). Coincidentally, fortuitous freeze-fracture images that show the tops of the stereocilia also revealed three indentations, spaced ∼20–30 nm apart, which apparently corresponded to the insertions of the three branches into the membrane (Fig. 3E). Large intermem-
brane particles have been previously localized near this region in cochlear hair cells (15).

**Bending and Stretching of Tip Links.** In 13 of the 58 tip links examined, we observed tip links that appeared to be under compression (Fig. 4A). In all cases, the tip links appeared smoothly buckled and without sharp bends, indicating high flexural rigidity. Similar behavior was observed in thin-section TEM images (Fig. 4D). These samples were subjected to chemical fixation; buckling observed therefore might arise from increased flexural rigidity afforded by fixation. Nevertheless, because fixation should immobilize adjacent stereocilia and prevent them from sliding along each other and stressing the tip links, tip-link buckling more likely reflects the structure in an unfixed state.

We frequently saw images of tip links that appeared tensed, apparently pulling the membrane of the shorter stereocilium away from the underlying cytoskeleton (Fig. 4B and C). This phenomenon, known as tenting, also has been observed by Kachar et al. (3). Thin-section TEM image of a tip link in a relaxed state. (Scale bars = 100 nm.)

**Sensitivity of the Tip Link to Various Chemical Disruption.** To gain further insight into their composition and properties, we examined chicken basilar papilla tip links for susceptibility to cations, sugars, proteases, and other agents. Many had no significant effects on tip links (Table 1). One protease previously reported to sever tip links, elastase (7), was ineffective unless we pre-treated papillae with N-glycosidase F; chondroitinase ABC accentuated this effect (Table 1). Tip links remained largely intact after treatment with saline solutions buffered from pH 4 to 10 (Fig. 5A). By contrast, tip links were eliminated after exposure to 1 mM La3+ or Tb3+ (Table 1). After tip links were

**Table 1. Effect of disruptive agents on tip links**

| Agent | Score |
|-------|-------|
| 1 mM LaCl3 | − (4) |
| 5 mM LaCl3 | − (2) |
| 1 mM TbCl3 | + / − (3) |
| 5 mM TbCl3 | − (1) |
| 20 mM BaCl2 | + / − (3) |
| 10 mM sodium periodate | + / − (8) |
| 25 mM mannose-6-phosphate | + / − (4) |
| 10 mM acetyleneuraminic acid | + / − (2) |
| 1 mg/ml elastase, 200 units/ml N-glycosidase F | + / − (3) |
| 1 mg/ml elastase, 10 units/ml chondroitinase ABC, 200 units/ml N-glycosidase F | − (5) |
| 1 mg/ml chymotrypsin, 200 units/ml N-glycosidase F | + / − (3) |
| 1 mg/ml chymotrypsin, 10 units/ml chondroitinase ABC, 200 units/ml N-glycosidase F | − (5) |

Basilar papillae were treated for 10 min with the indicated reagents. For combinations of glycosidases (N-glycosidase F and chondroitinase) and proteases, papillae were first treated with glycosidases for 30 min, followed by a wash and a 10-min protease treatment. Scores: (+), 50–100% number of tip links as control; (+ / −), 15–50% of control; (−), 0–15% of control; number of papillae examined in parentheses. The following were scored (+) with one or two papillae each: 1 mM BaCl2, 1 mM CoCl2, 5 mM MnCl2, 10 mM galactose, 10 mM mannose, 10 mM lactose, 10 mM fucose, 10 mM mannose-1-phosphate, 25 mM fucose-1-phosphate, 10 mM N-acetyl-d-galactosamine, 10 mM N-acetyl-d-glucosamine, 1 mM methyl-N-acetylmannopyranoside, 5 mM heparin, 1 mg/ml chondroitin sulfate, 100 μg/ml fucoidan, 200 units/ml N-glycosidase F, and 10 units/ml chondroitinase ABC. The following were scored (+) with one or two papillae each at 0.5 mg/ml and 1 mg/ml: trypsin, chymotrypsin, papain, bromelain, ficin, protease K, S. aureus V8 protease, and elastase.

![Fig. 4. Response of the tip link to compressive and tensile forces.](image)

![Fig. 5. Response of tip links to chemical treatment.](image)
Polymer that remains intact during large bundle displacements, an actin filament is actin can be strong; for example, the force required to rupture of two extended chains. Double-stranded, helical polymers like polypeptides, either as a polymer of many subunits or as a dimer of two extended chains. This portion of the tip link must contact each other as a single α-helix, a coiled-coil, or a triple helix, form structures too narrow to be tip links. The tip link could be similar to other elongated proteins known to be elastic, such as titin (≈0.7 mN m⁻¹ per 150 nm; ref. 28) and tenascin (≈2 mN m⁻¹ per 150 nm; ref. 29); titin and tenascin, however, do not helically dimerize. Although other elongated extracellular proteins do dimerize, they do usually do so in an antiparallel orientation (30, 31); by contrast, the asymmetry of the tip-link structure strongly suggests a parallel orientation of the protofilaments. Interestingly, Ig tandem domains dimerize in parallel, both in Fc and Fab interactions (32).

Polymer models for the tip link face several challenges, however. Tip-link length appears relatively uniform within a given bundle or organ and across species (3–6, 21); a template therefore would be required to permit precise control of filament length. In addition, the bifurcation of the tip link into two strands creates a problem. A double-stranded helical structure like actin is stable because monomers can interact both along the protofilament axis and between protofilaments (27). If the protofilaments of a polymeric helical structure could be disentwined, they would rapidly disintegrate. Depending on how the branches of the tip link connect with the membrane, however, they may have to withstand forces up to half as great as the helical section of the gating spring.

The tip link also might be constructed of two extended polypeptide filaments, helically coiling around each other. Familiar elongated and coiled protein structures, such as a single α-helix, a coiled-coil, or a triple helix, form structures too narrow to be tip links. The tip link could be similar to other elongated proteins known to be elastic, such as titin (≈0.7 mN m⁻¹ per 150 nm; ref. 28) and tenascin (≈2 mN m⁻¹ per 150 nm; ref. 29); titin and tenascin, however, do not helically dimerize. Although other elongated extracellular proteins do dimerize, they do usually do so in an antiparallel orientation (30, 31); by contrast, the asymmetry of the tip-link structure strongly suggests a parallel orientation of the protofilaments. Interestingly, Ig tandem domains dimerize in parallel, both in Fc and Fab interactions (32).

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The relatively large diameter of the tip link makes it highly unlikely that it is composed of polysaccharide or polynucleotide chains. This portion of the tip link therefore must be formed by polypeptides, either as a polymer of many subunits or as a dimer of two extended chains. Double-stranded, helical polymers like actin can be strong; for example, the force required to rupture an actin filament is ≈100 pN (25). If the tip link is a helical polymer that remains intact during large bundle displacements, the large number of monomer-monomer contacts required for strength would likely render the filament very stiff; for example, an actin filament with the length of a tip link would have a stiffness of ≈300 mN m⁻¹ (26), much larger than that of the gating spring.

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portion, it must be nearly inextensible. If a double filament constitutes the extended portion, it is still likely to be much stiffer than paired 150-nm titin or tenascin molecules, which already would be stiffer than the gating spring.

Implications for Models of Transduction. Active transduction channels apparently can be found near either end of a tip link (34). If the tip link directly gates channels at both ends, the link’s asymmetric structure implies that the channel-tip link connection differs at the two ends. Alternatively, transduction channels may be indirectly coupled or gated by structures other than tip links (35).

If tip links are rigid, they could convey compressive forces to transduction channels until buckling. If the tip link lacks a hinge (or other flexible region (a correct assumption if images such as those in Fig. 4 reflect properties of the unfixed link), the force required to buckle the tip link (F) is given by $F = \pi^2 Y l^2$, where $YI$ is the flexural rigidity, and $L$ is the tip link length (Fig. 6). If the flexural rigidity of the tip link resembles that of actin, $\approx 10^{-25}$ Mm$^2$ (36), the buckling force would be $\approx 40$ pN; an inhibitory displacement of $\approx 250$ nm in bullfrog saccular hair bundle structures could provide this force. By contrast, if its flexural rigidity is similar to that of the muscle-protein titin, $10^{-28}$ Mm$^2$ (28), only $\approx 40$ F will be required to buckle the tip link. Measured under the same conditions, however, titin molecules are narrower than the tip link (37). The tip-link buckling force is therefore likely to be considerably higher than the lower value. Although saturating channel-closure kinetics, decreased bundle stiffness for negative displacements, and adaptation rates for negative steps all imply that gating springs can slacken (2), rigid tip links may transmit compressive forces once the gating springs have slackened.

The proposed inextensibility of the tip link suggests that another elastic element within the hair bundle is the true gating spring, and that the tip link instead connects to it in series to direct force to the channel (2, 4); alternatively, elasticity could be shared among several components, perhaps including the tip link. Although rigorous measurement of tip-link length and separation of the membrane from the tip density during mechanical stimuli would address this issue directly, these experiments are extremely difficult with thin-section TEM and, because of the projection problem mentioned above, are especially difficult with freeze-etch microscopy. The tip link appears too rigidly attached to the cytoskeleton at the taller stereocilium for an elastic element to be present there. By contrast, the membrane of the shorter stereocilium often is tented away from the underlying cytoskeleton and tip density, suggesting that an elastic element might be located there. Membrane bilayers are probably far too compliant to serve as the gating spring (38). Instead, we suggest that intracellular filaments connecting the tip density to the membrane (17) are better candidates to form the gating spring (Fig. 6).

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