Caprin-1 is a target of the deafness gene *Pou4f3* and is recruited to stress granules in cochlear hair cells in response to ototoxic damage

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Accepted 9 November 2010
Journal of Cell Science 124, 1145-1155

doi:10.1242/jcs.076141

Summary

The POU4 family of transcription factors are required for survival of specific cell types in different sensory systems. Pou4f3 is essential for the survival of auditory sensory hair cells and several mutations in human *POU4F3* cause hearing loss. Thus, genes regulated by Pou4f3 are likely to be essential for hair cell survival. We performed a subtractive hybridisation screen in an inner-ear-derived cell line to find genes with differential expression in response to changes in Pou4f3 levels. The screen identified the stress-granule-associated protein Caprin-1 as being downregulated by Pou4f3. We demonstrated that this regulation occurs through the direct interaction of Pou4f3 with binding sites in the *Caprin-1* 5′ flanking sequence, and describe the expression pattern of *Caprin-1* mRNA and protein in the cochlea. Moreover, we found Caprin-1-containing stress granules are induced in cochlear hair cells following aminoglycoside-induced damage. This is the first report of stress granule formation in mammalian hair cells and suggests that the formation of Caprin-1-containing stress granules is a key damage response to a clinically relevant ototoxic agent. Our results have implications for the understanding of aminoglycoside-induced hearing loss and provide further evidence that stress granule formation is a fundamental cellular stress response.

Key words: Stress granules, Hair cells, Aminoglycoside, Cochlea, Hearing

Introduction

The POU4 domain transcription factors are well-established regulators in sensory systems. Pou4f1 and Pou4f2 have overlapping, but distinct, expression patterns in retinal ganglion cells and sensory neurons (Badea et al., 2009; Ninkina et al., 1993; Xiang et al., 1995). By contrast, the expression of Pou4f3 (also known as Brn-3c and Brn3.1) is restricted to discrete subsets of sensory neurons (Ninkina et al., 1993) and is crucial for inner ear development; it is the only POU4 transcription factor present in the sensory receptor hair cells of the cochlea (Xiang et al., 1997). In *Pou4f3*-null mice, hair cells fail to mature and undergo apoptosis during early postnatal development, resulting in mice with auditory or vestibular dysfunction (Erkman et al., 1996; Xiang et al., 1997; Xiang et al., 1998). In addition, mutations in human *POU4F3* result in adult-onset non-syndromic hearing loss (DFNA15) (Collin et al., 2008; Pauw et al., 2008; Vahava et al., 1998; Weiss et al., 2003).

Hair cells are the mechanoreceptors for sound, head motion and gravity (Pickles and Corey, 1992). Unlike in a number of vertebrate classes, including birds, amphibians and fishes, mammals are unable to regenerate these essential cells once they are lost (Edge and Chen, 2008). Hair cell damage and death are the predominant pathologies underlying many types of acquired hearing loss, including noise-induced, ototoxin-induced and age-related hearing loss (Francis et al., 2003; Rizzi and Hirose, 2007; Wang et al., 2002). Despite their clinical importance, the pathways regulating hair cell survival remain largely unknown. Identification of Pou4f3 targets in hair cells is likely to reveal hair cell pathways or genes that might also have relevance in other systems, and might identify new pathological mechanisms or therapeutic targets. To date, there is evidence that Pou4f3 regulates neurotrophin gene expression (Clough et al., 2004); the two transcription factors Gfi-1 and Lhx3 are also known to be dysregulated in *Pou4f3* mutant mice and are presumed to be indirect downstream targets of Pou4f3 (Hertzano et al., 2007; Hertzano et al., 2004). Identifying Pou4f3 targets by comparing gene expression between cochlear tissue from wild-type and mutant Pou4f3 mice has limitations because of the complication of ongoing hair cell death (Xiang et al., 1997). Therefore, to find additional novel targets of Pou4f3, we manipulated levels of Pou4f3 expression in an inner ear cell line (OC-2 cells) and performed a subtractive hybridisation screen.

This screen identified Caprin-1 (cytoplasmic activation and proliferation-associated protein-1) as a target of Pou4f3 regulation. Caprin-1 (also called RNG105) is a cytoplasmic phosphoprotein that is known to be highly expressed in the thymus, spleen and brain (Grill et al., 2004; Wang et al., 2005). In the brain, Caprin-1 is localised to RNA granules in postsynaptic dendrites of hippocampal neurons and it might function to regulate localised translation (Shiina et al., 2005). Caprin-1 associates with another form of RNA granule in cell lines: cytoplasmic stress granules (cytoplasmic aggregates that regulate translation of subsets of mRNA following exposure to environmental stress). Caprin-1 has been shown to be a component of the stress granules induced by arsenite treatment in HeLa cells and exogenous expression of Caprin-1 protein alone is sufficient to induce stress granule formation in these cells (Solomon et al., 2007). Here we describe, for the first time, *Caprin-1* regulation by Pou4f3, and examine the expression patterns of *Caprin-1* mRNA and protein in the cochlea. In addition, we characterise stress granule formation and the parallel changes in *Caprin-1* expression during ototoxic

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aminoglycoside damage in a mammalian cochlear-explant-based model of cellular stress. Our data suggest a model in which hair cell damage modifies Pou4f3 activity, resulting in an increase in Caprin-1 expression and, thus, stress granule formation. Identification of these mechanisms, therefore, has implications for our understanding of how ototoxins, such as aminoglycoside antibiotics, induce hearing loss. Furthermore, a link between POU4 transcription factor regulation and the stress granule response might be relevant in other systems.

Results

Identification of *Caprin-1* as a downstream target of Pou4f3

To identify Pou4f3 target genes, we used the OC-2 cell line, which is derived from the immortomouse inner ear sensory epithelium at embryonic day 13. OC-2 cells show expression of both supporting cell and hair cell markers, including Pou4f3, and are commonly used as an in vitro system to study the auditory sensory epithelium (Rivolta and Holley, 2002). We manipulated endogenous Pou4f3 levels in OC-2 cells (cultured under proliferating conditions) by transfecting them with either a Pou4f3 expression vector or an antisense Pou4f3 construct. These cells were then used in a subtractive hybridisation screen to identify a pool of candidate target genes that had differential expression in response to altered levels of Pou4f3. The subtractive hybridisation screen was performed in both forward and reverse directions to identify both positively and negatively regulated genes. Clone A10 was shown, through serial virtual northern analysis, to be a highly enriched transcript in the reverse-subtracted cDNA library, thus confirming that this gene had increased expression in OC-2 cells with reduced Pou4f3 levels (Fig. 1A, clone A10). Sequencing of clone A10 and subsequent BLAST analysis of the Ensembl mouse genome

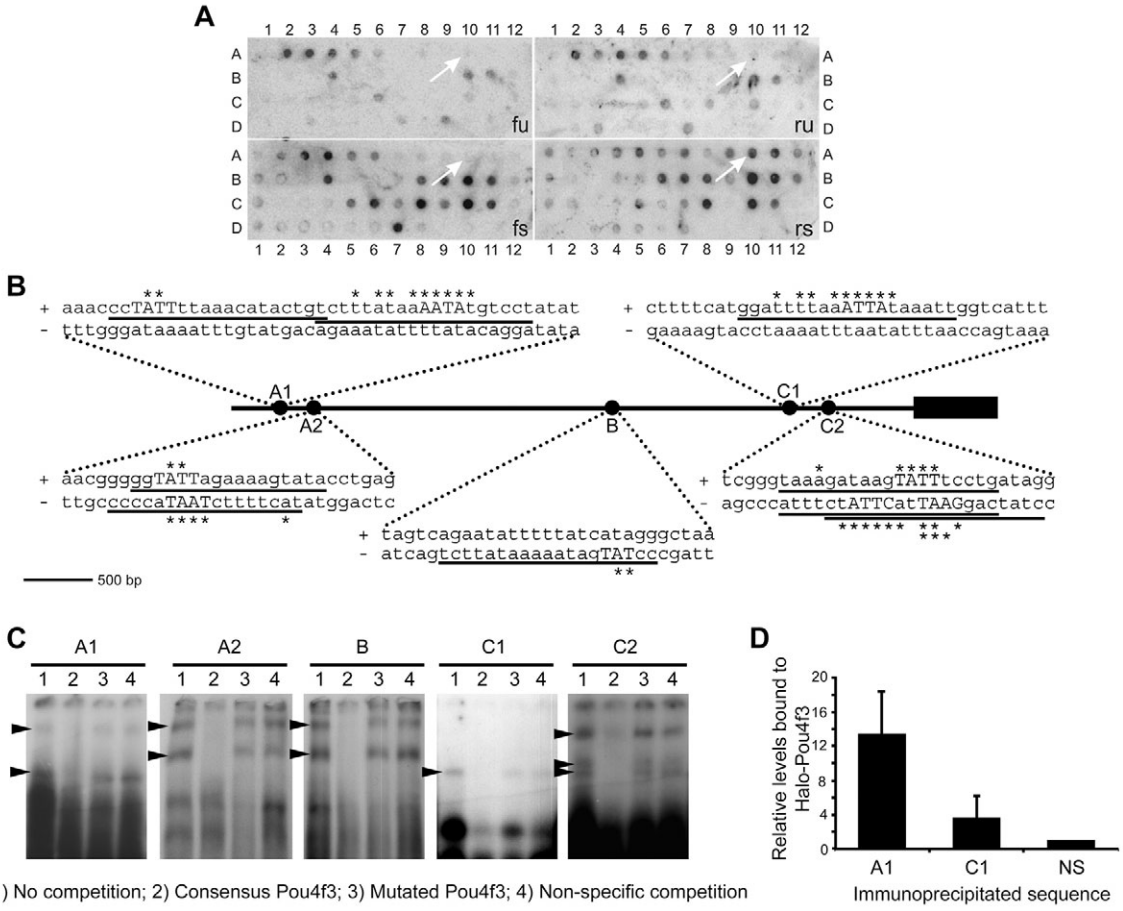

Fig. 1. Identification of *Caprin-1* as a Pou4f3 target gene. (**A**) Four replicate clone arrays obtained from the reverse subtraction experiment. Replicate dot blots were hybridised with radiolabelled cDNA from forward (increased Pou4f3 minus decreased Pou4f3) and reverse (decreased Pou4f3 minus increased Pou4f3) experiments, with both subtracted and unsubtracted cDNA. fu, forward unsubtracted; fs, forward subtracted; ru, reverse unsubtracted; rs, reverse subtracted. The *Caprin-1* clone at position A10 on each filter (arrows) is highly enriched in the reverse subtracted cDNA, suggesting that it is downregulated by Pou4f3. (**B**) Putative Pou4f3-binding sites used as EMSA probe sequences (A1, A2, B, C1 and C2) are shown. Underlined bases correspond to sequences identified by MatInspector, matches to the core sequence are shown in capital letters, and the asterisk indicates nucleotides with a matrix position conservation (Ci) value of >60/100. (**C**) EMSA analysis. OC-2 cell nuclear extract was incubated with the radiolabelled probes shown in B. These were incubated alone (lane 1), or with a 250-fold excess of the indicated unlabelled competitor sequences (lanes 2–4). Bound protein is indicated (arrowheads). (**D**) ChIP analysis. Binding of Halo-tagged Pou4f3 to sites A1 and C1, and to a control sequence (NS) within the *Caprin-1* 5′ flanking sequence, in native OC-2 cell chromatin as determined by qPCR. Levels are plotted relative to the levels bound by a control vector after 40 cycles; however, the control sequence was not detected in all three replicates, therefore the minimum possible fold differences are shown on the basis of an assigned cycle threshold of 40, rather than the absolute threshold. Other sites were not precipitated above background levels. Error bars represent 95% confidence intervals (n=3).

identified a 99.69% match, over 644 bp, to the 3′ untranslated region (UTR) of the *Caprin-1* gene (ENSMUSG00000027184).

Caprin-1 expression has been characterised in various cell types, including HeLa cells, NIH 3T3 mouse fibroblasts and hippocampal neurons (Shiina et al., 2005; Solomon et al., 2007), but not in the inner ear or inner-ear-derived cells. Immunolabelling with an anti-Caprin-1 antibody showed expression of the endogenous Caprin-1 protein in the cytoplasm of OC-2 cells, confirming that Caprin-1 is expressed in the inner-ear-derived OC-2 cell line used in the subtractive hybridisation screen (supplementary material Fig. S1).

Predicted Pou4f3-binding sites in the *Caprin-1* 5′ flanking sequence are able to bind Pou4f3 in EMSA and ChIP analyses

We analysed the sequence upstream of the *Caprin-1* gene to investigate whether its regulation by Pou4f3 is through a direct interaction with binding sites in the *Caprin-1* promoter. Genes2Promoter software (Genomatix Software) was used to predict the region containing the mouse *Caprin-1* promoter sequence and a 5-kb region was analysed with MatInspector to identify potential Pou4f3-binding sites. From multiple predicted sites, five were selected for functional analysis: the three sites with the highest stringency predictions and two lower stringency matches that contained two overlapping predicted sites (Fig. 1B).

The ability of Pou4f3 to bind to these five predicted sites in the *Caprin-1* 5′ flanking sequence was tested in electrophoretic mobility-shift assays (EMSAs). Endogenous OC-2 nuclear proteins bound to each of the five sequences selected, causing shifted bands in EMSA analysis (Fig. 1C). Incubating the reaction with a 250-fold excess of an unlabelled consensus Pou4f3-binding site competed for the binding, and abolished one or more shifts, for each of the five predicted sequences tested. Inclusion of a mutated Pou4f3-binding site or an unrelated binding site had no effect on binding. All five sites also bound in-vitro-translated Pou4f3 (data not shown).

To test whether these interactions also take place within cells, we performed chromatin immunoprecipitation (ChIP) analysis. Chromatin was isolated from OC-2 cells transfected with Halo-tagged Pou4f3 and, following precipitation using HaloLink Resin, the amounts of the five binding sites were compared by quantitative real-time PCR (qPCR). Of the five sites, A1 and C1 were precipitated above background levels, with A1 being preferentially precipitated (Fig. 1D), indicating that both sites are capable of binding Pou4f3 in the context of native chromatin in OC-2 cells. Although sites A2, B and C2 could bind Pou4f3 in vitro, we did not detect any binding above background levels in ChIP analyses, suggesting that they might not act as functional Pou4f3-binding sites in situ.

The *Caprin-1* 5′ flanking sequence contains a promoter that is repressed by Pou4f3

To determine whether Pou4f3 can regulate *Caprin-1* promoter activity through any of the five putative Pou4f3-binding sites, a 4.7-kb region upstream of the predicted transcriptional start site, containing all five sites, was cloned from mouse genomic DNA into a promoterless luciferase vector to create 4.7kb-*Cap1*-LUC (Fig. 2A). 4.7kb-*Cap1*-LUC activity was ~200-fold higher than that of the empty vector in transfected OC-2 cells, indicating it contains a promoter capable of driving transcription. In co-transfection studies, there was a dose-dependent decrease in 4.7kb-*Cap1*-LUC activity when the levels of Pou4f3 were increased, resulting in a 65% reduction with the maximum amount of Pou4f3 (Fig. 2B). This effect was due to active repression by Pou4f3 protein because co-transfection of equivalent levels of cDNA encoding a non-DNA-binding mutant Pou4f3 (dreidel, ddl) (Hertzano et al., 2004) did not decrease promoter activity (Fig. 2B). Taken together, these results suggest that the *Caprin-1* 4.7-kb 5′ flanking region contains a functional promoter with sequences that confer negative regulation of promoter activity by Pou4f3, consistent with its detection in the reverse-subtracted library of our screen.

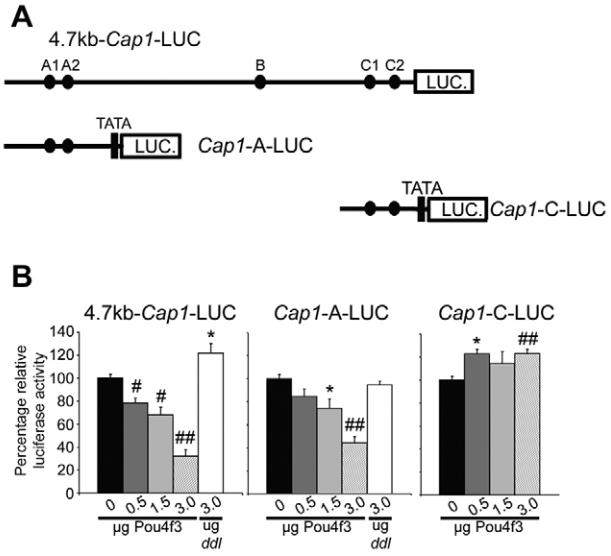

Fig. 2. The *Caprin-1* 5′ flanking region is regulated by Pou4f3. (**A**) Schematic showing the luciferase reporter constructs containing the *Caprin-1* 5′ flanking sequence used in the co-transfection experiments shown in B. Each construct was co-transfected into OC-2 cells with either a Pou4f3 or mutant Pou4f3 *Driedel* (*ddl*) expression vector. (**B**) Dual luciferase assays were used to determine promoter activity, which is expressed as a percentage of promoter induction relative to activity with the empty expression vector. ug, µg of expression vector. Error bars represent s.e.m, (n=6). Student's t-tests were performed comparing each condition with 0 µg of Pou4f3 expression vector. *P<0.05; #P<0.005; ##P<0.001.

To determine the relative contribution of the five putative sites to Pou4f3-mediated repression of *Caprin-1* promoter activity, a 1.2-kb sequence, containing sites A1 and A2, and an 840-bp sequence, containing sites C1 and C2, were each inserted upstream of the TATA-box element in pGL4.23 (Fig. 2A, *Cap1*-A-LUC and *Cap1*-C-LUC, respectively). The effect of expressing increasing amounts of Pou4f3 on the activity of the promoter constructs was tested in OC-2 cells; *Cap1*-A-LUC, but not *Cap1*-C-LUC, showed a reduction in luciferase activity, with the maximum amount of Pou4f3 resulting in a decrease of 55% (Fig. 2B). This reduction is almost equivalent to that with 4.7kb-*Cap1*-LUC, and *Cap1*-A-LUC activity was also unaffected by expressing ddl (Fig. 2B), suggesting that Pou4f3-binding sites within the inserted sequence confer repression on the heterologous promoter.

Exogenous expression of Caprin-1 induces stress granule formation in OC-2 cells

Previous studies, in a variety of cell lines, have shown that Caprin-1 is a component of both RNA transport granules and cytoplasmic stress granules, being able to bind specific mRNAs and potentially regulate their translation (Shiina et al., 2005; Solomon et al., 2007). To determine the role of Caprin-1 in inner-ear-derived cells, we transfected an expression vector encoding GFP-tagged Caprin-1

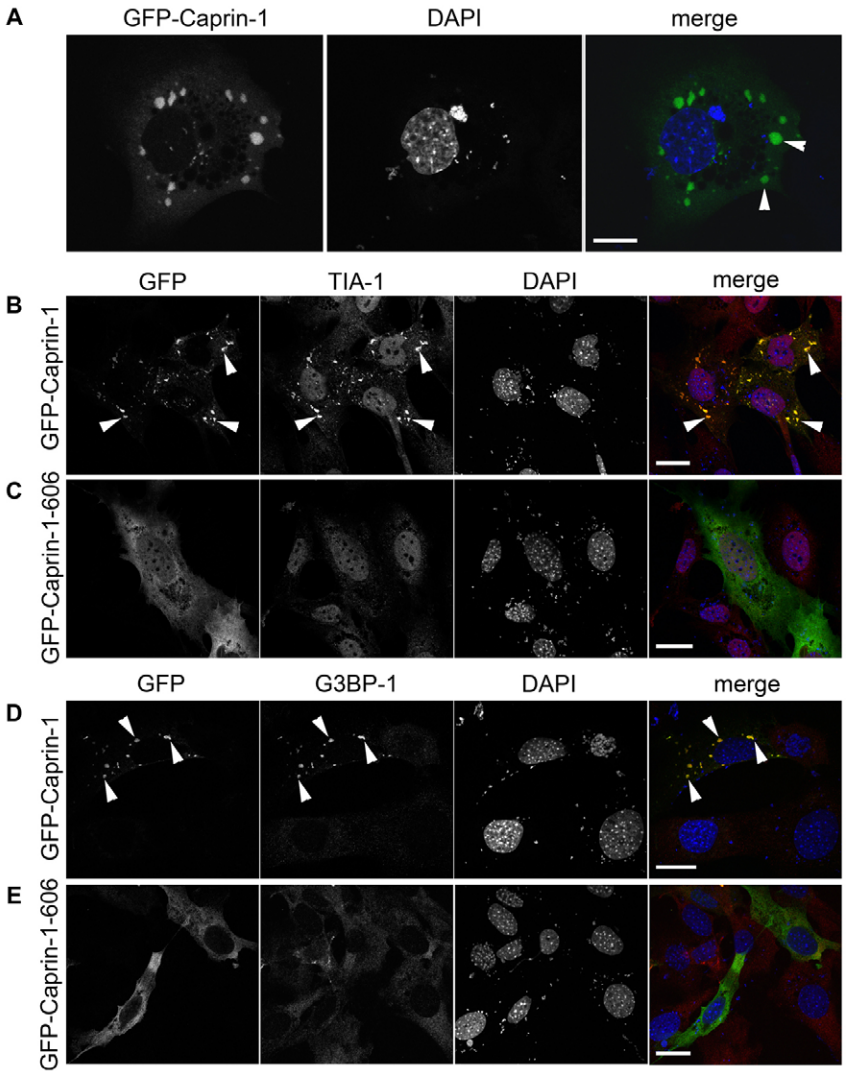

Fig. 3. Exogenous Caprin-1 induces formation of cytoplasmic granules that colocalise with the stress granule marker TIA-1 in OC-2 cells. (**A**) Exogenous Caprin-1 expression (green) induced formation of cytoplasmic granules (arrowheads) in OC-2 cells fixed 24 hours after transfection with GFP–Caprin-1. (**B**,**D**) Granules formed by exogenous Caprin-1 also label with the stress granule markers TIA-1 (B) and G3BP-1 (D) (red, arrowheads). (**C**,**E**) Overexpression of truncated Caprin-1 (GFP–Caprin-1-606, red) did not induce stress granule formation in OC-2 cells. Nuclei were stained using DAPI (blue). Scale bars: 20 µm.

(GFP–Caprin-1) (Solomon et al., 2007) in OC-2 cells. In ~25% of transfected cells, aggregates of GFP–Caprin-1 were observed in the cytoplasm (Fig. 3A); these were similar in appearance to cytoplasmic stress granules found in experiments performed in HeLa cells (Solomon et al., 2007). We noted that the GFP fluorescence signal was generally lower in GFP–Caprin-1-positive cells that did not contain GFP aggregates. This suggests that the granules formed in cells where exogenous Caprin-1 was present at higher levels, and indicates a threshold effect. Using live-cell confocal time-lapse imaging of transfected cells, granules were shown to exhibit dynamic behaviour, moving throughout the cytoplasm from the perinuclear region to relatively distant extended processes away from the cell body. As granules moved throughout the cell they frequently coalesced into larger aggregates, which then continued to exhibit dynamic behaviour (supplementary material Movie 1). Large aggregates were also seen to disperse into multiple smaller granules, such that granule number and size varied substantially over time within a single cell, typically ranging from 2–3 clusters of 4–5 µm in diameter to numerous smaller granules of <1 µm in diameter (supplementary material Movie 2).

To confirm whether these aggregates were stress granules, we labelled GFP–Caprin-1-transfected OC-2 cells with an antibody against TIA-1 (T-cell-restricted antigen 1), a known stress granule marker (Anderson and Kedersha, 2002a). TIA-1 was observed in the nucleus of OC-2 cells, where it is located in normal conditions, and also colocalised with the GFP–Caprin-1-positive granules (Fig. 3B), where present. No GFP- and TIA-1-positive stress granules were observed in cells expressing a GFP-labelled truncated Caprin-1 (GFP–Caprin-1-606; Fig. 3C), which lacks the C-terminal RNA-binding motif required for stress granule formation (Solomon et al., 2007), despite similar levels of transfection (as estimated by the levels of GFP expression). Expression of GFP–Caprin-1-606 was comparatively diffuse throughout the cytoplasm of OC-2 cells. Similar data were obtained using an antibody against G3BP-1, another stress granule marker (Tourriere et al., 2003); G3BP-1 also colocalised with the GFP–Caprin-1-positive granules but not with GFP–Caprin-1-606 (Fig. 3D,E). These results suggest that, in OC-2 cells, as in HeLa cells, exogenous expression of Caprin-1 is sufficient to induce the formation of stress granules, and that this induction requires the C-terminal RNA-binding motif.

Caprin-1 is expressed at different levels in hair cells and supporting cells in the rodent inner ear

To analyse Caprin-1 expression in the mammalian inner ear sensory organ for the first time, we examined *Caprin-1* mRNA expression in the P18 mouse cochlea and utricle by qPCR, and compared this

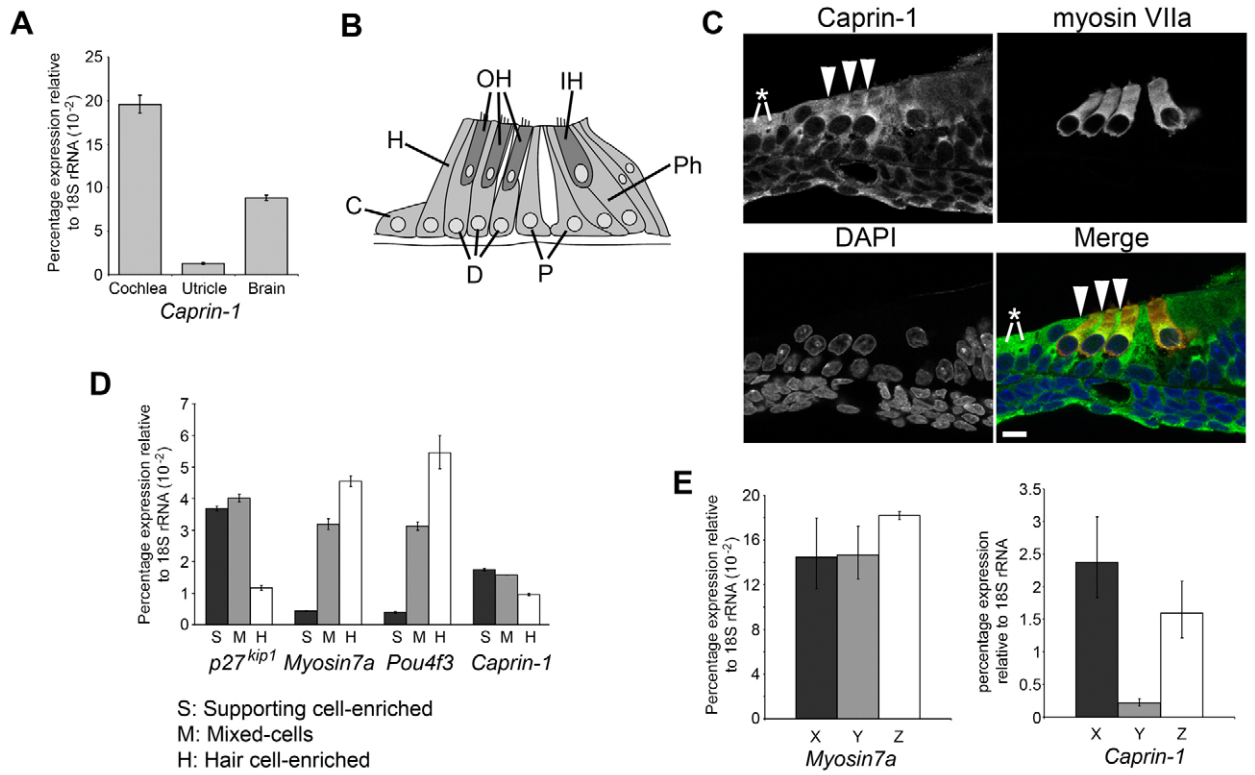

Fig. 4. Caprin-1 is expressed in the inner ear in both supporting cells and hair cells. (**A**) Levels of *Caprin-1* and *Pou4f3* mRNA were determined by qPCR in mouse cochlea, utricle and brain. For all qPCR experiments, 18S rRNA was used as an endogenous control and expression levels were calculated relative to this endogenous control. Error bars represent 95% confidence intervals. (**B**) Schematic showing the arrangement of cells within the organ of Corti. OH, outer hair cells; IH, inner hair cells; D, Deiters' cells; H, Hensen's cells; C, Claudius' cells; P, pillar cells; and Ph, phalangeal cells. (**C**) Vibratome slices through a P2 rat cochlea show Caprin-1 (green) expression in cells in the sensory epithelium (the organ of Corti), the hair cell marker myosin VIIa (red) and nuclei stained with DAPI (blue). Arrowheads indicate greater Caprin-1 expression in phalangeal processes of the Deiters' cells that intercalate between hair cells. The asterisks indicate expression in Claudius' cells. Scale bar: 10 µm. (**D**) Samples enriched for particular cell types (supporting-cell-enriched, hair-cell-enriched and a mixed-cell sample of Deiters' and hair cells) were collected and analysed by qPCR. Expression levels of $p27^{kip1}$, *Myo7a*, *Pou4f3* and *Caprin-1* were calculated relative to the endogenous control. $p27^{kip1}$ and *Myo7a* levels indicate the degree of enrichment for supporting cells and hair cells, respectively. *Pou4f3* expression is highest in the hair-cell-enriched pool, whereas *Caprin-1* was lowest in the hair-cell-enriched pool. (**E**) Single-cell qPCR. *Caprin-1* levels were measured in individual hair cell samples (X, Y and Z) that expressed *Myo7a* but not $p27^{kip1}$. These cells were collected from the same explant culture, but are representative of results obtained from more than five cultures.

with the expression in the brain (Fig. 4A), where Caprin-1 protein is expressed in neuronal cells (Shiina et al., 2005). *Caprin-1* was expressed in all three tissue types, having the highest relative level of expression in the cochlea, where expression was over 15-fold higher than in the utricle and 2.2-fold higher than in the brain.

A major limitation when working with cells of the inner ear is the difficulty in isolating pure cell populations from these complex multicellular organs (see Fig. 4B) in order to be able to determine cell-specific gene expression. Immunofluorescence analyses in cochlear slices indicated that Caprin-1 was expressed in the Pou4f3-containing sensory hair cells, but that it was also expressed in some supporting cells, in particular the Deiters' cells, which surround the outer hair cells, and Claudius' cells (Fig. 4C). Therefore, any attempt to quantify hair cell *Caprin-1* expression using whole cochlear tissue is compromised by the contribution from supporting cells. Previously, other researchers have used isolated sensory epithelia to quantify gene expression (Hu et al., 2009), although these preparations still include approximately ten different cell types, of which hair cells are a minority. To document gene expression in separate cell types, we used micropipette aspiration of specific cell populations from early postnatal rat cochlear explants. Using this approach we collected samples that were highly enriched for a particular cell type. Cell-specific markers, myosin-VIIa (*Myo7a*) for hair cells and $p27^{kip1}$ (also known as *Cdkn1b*) for supporting cells, were used to establish the degree of enrichment of the different cell types in the samples collected (Fig. 4D) [$p27^{kip1}$ is not expressed in hair cells in the neonatal or adult cochlea (Chen and Segil, 1999)]. Three samples were collected: a hair-cell-enriched sample, a mixed-cell sample of hair cells and Deiters' cells (the nearest neighbour cell to the outer hair cells), and a supporting-cell-enriched sample (containing predominantly Claudius' and Hensen's cells). Lower levels of $p27^{kip1}$ were present in the hair-cell-enriched sample than in the supporting-cell-enriched and mixed-cell samples (Fig. 4D). *Pou4f3* acted as a second hair-cell-specific marker and its expression paralleled that of *Myo7a*, with highest relative expression in the hair-cell-enriched sample. These results confirmed the enrichment of the specific cell populations in the samples. *Caprin-1* expression was lower in the hair-cell-enriched sample, compared with the other two samples, although it was not as low as might be predicted if *Caprin-1* was expressed in the supporting cells alone (Fig. 4D). These results parallel the immunofluorescence data and indicate that *Caprin-1* is expressed in hair cells, although the levels

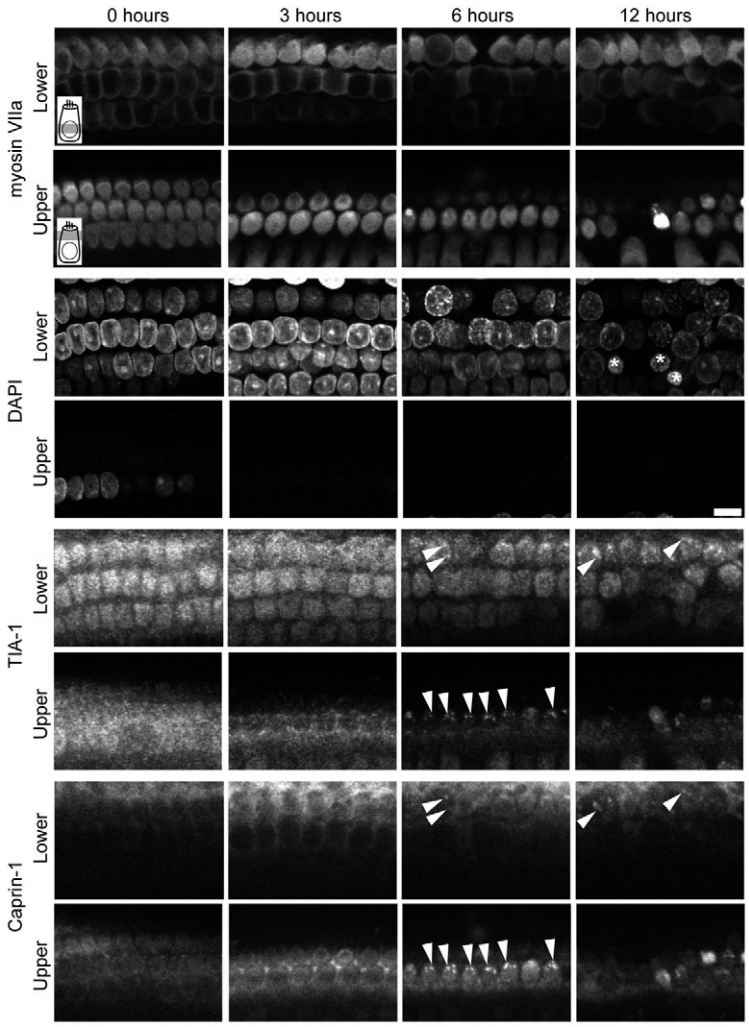

Fig. 5. Changes in TIA-1 and Caprin-1 expression during neomycin treatment. Cochlear explant cultures from P2 rats were treated with 1 mM neomycin for 0, 3, 6 or 12 hours. Caprin-1, TIA-1 and myosin VIIa expression were determined by triple-label immunofluorescence, with DAPI to assess chromatin structure. Areas shown are from the basal end of cochlear coils and were selected without observing fluorescent signals, to avoid bias. Images are the averages of four confocal optical sections representing ~6-µm-optical-slices of the outer hair cell region. The upper and lower rows represent focal planes of the upper apical pole and lower nuclear pole of hair cells, respectively (see inset). Arrowheads indicate hair cell granules labelled with both Caprin-1 and TIA-1 (also see supplementary material Fig. S2). Asterisks indicate condensed nuclei. Scale bar: 10 µm (for all images).

of expression are lower than in supporting cells, which do not express *Pou4f3* (Fig. 4D).

Although data from the hair-cell-enriched samples give a good indication of hair cell gene expression, results are complicated by the influence of some cDNA from supporting cells in the sample (Fig. 4D, $p27^{kip1}$). Therefore, to improve the cell specificity further, we extended the qPCR approach to individual hair cells aspirated from cochlear explants, thereby reducing the opportunity for supporting cells to be accidentally aspirated from the sensory epithelium. Each sample was first analysed to establish whether it contained only hair cell cDNA, by measuring *Myo7a* and $p27^{kip1}$ expression. Subsequently, *Caprin-1* expression was determined in the single hair-cell samples that did not express $p27^{kip1}$ and, therefore, were not contaminated with supporting cells. *Caprin-1* levels were different in the three single-cell qPCR samples, but confirmed that *Caprin-1* is expressed in hair cells (Fig. 4E).

Mammalian hair cells respond to ototoxic damage by assembling stress granules containing Caprin-1

Work in cell lines has implicated Caprin-1 in stress granule formation but its role in native tissue and in vivo is unknown. A single previous report suggests that granule formation might play a part in the hair cell response to damage (Mangiardi et al., 2004). In that study, the aminoglycoside gentamicin, an ototoxic antibiotic known to specifically induce hair cell death and permanent hearing loss, triggers the formation of cytoplasmic granules in hair cells of the chick's hearing organ, the basilar papilla. Therefore, to examine whether stress granule formation also occurs in mammalian hair cells in response to damage, we treated cochlear explant cultures with the aminoglycoside neomycin. The in vivo base-to-apex gradient of aminoglycoside sensitivity persists in these cultures as described previously (Richardson and Russell, 1991; Lahne and Gale, 2008); therefore, the data presented are from the basal coil of the cochlear cultures. In untreated cultures, the highly organised structure of the organ of Corti (see Fig. 4B) could be clearly seen, indicated by the three regular rows of outer hair cells stained for the hair cell marker myosin VIIa (Fig. 5). Following neomycin treatment, hair cell loss occurs over time, with individual cells showing signs of damage while neighbouring cells appear relatively normal. In most experiments, hair cell loss began between 6 and 12 hours, by which time the orderly arrangement of hair cells was significantly disrupted, with gaps appearing in the rows of nuclei, and condensed nuclei were visible, indicating cell death (Fig. 5).

Stress granule formation in neomycin-treated cochlear explants was determined by examining TIA-1 localisation. In untreated tissue, TIA-1 colocalised with DAPI in the hair cells and surrounding supporting cells (Fig. 5, 0 hours); however, after 6 hours of neomycin treatment, granular concentrations of TIA-1

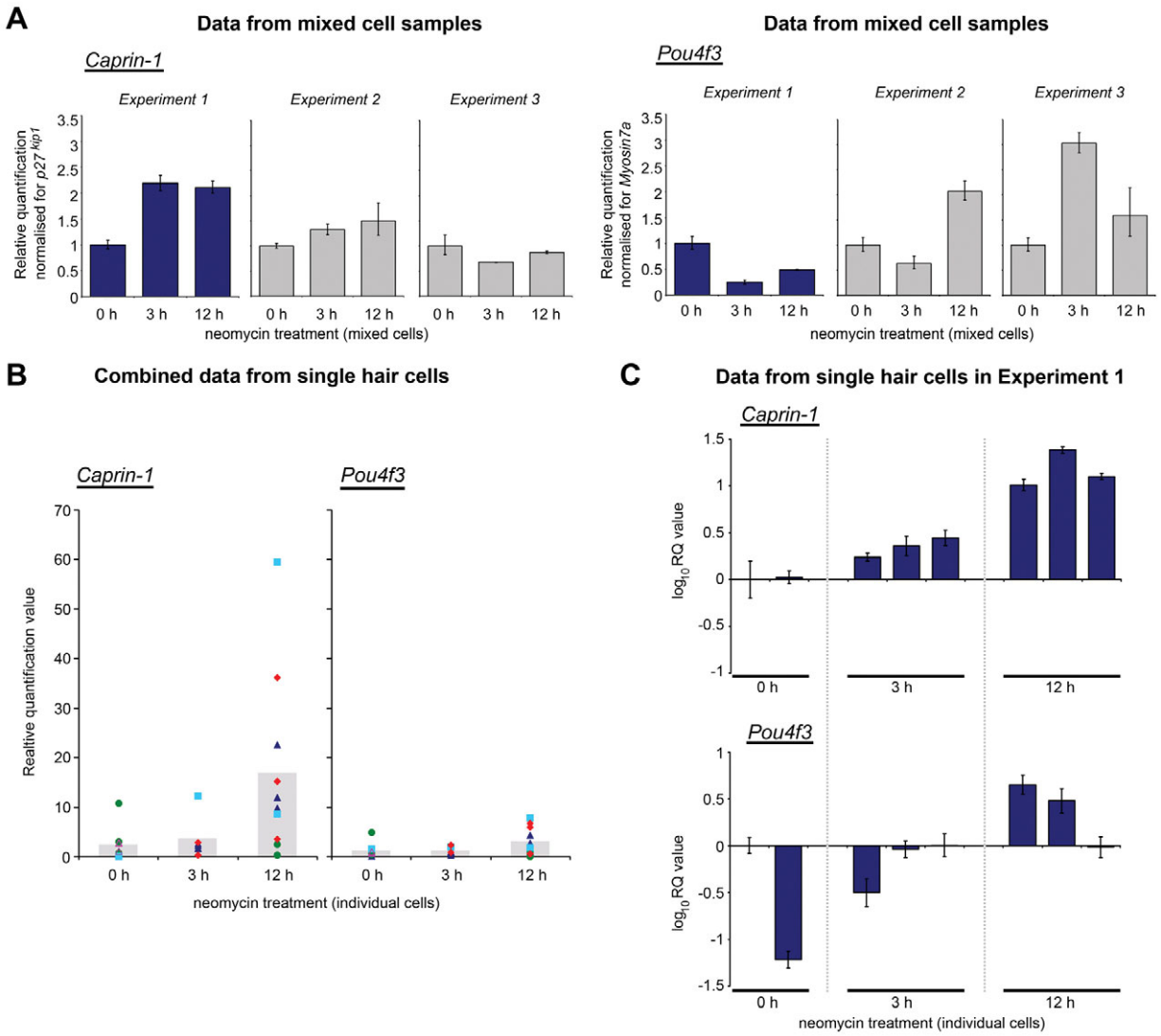

Fig. 6. Determining *Caprin-1* mRNA expression in hair cells following neomycin treatment. (**A**) Mixed-cell samples of outer hair cells and Deiters' cells were collected from cochlear explant cultures treated with 1 mM neomycin for 0, 3 or 12 hours, in three separate experiments. *Caprin-1* and *Pou4f3* mRNA expression levels were calculated relative to the expression at 0 hours and normalised to expression of the cell markers $p27^{kip1}$ and *Myo7a*, respectively. This adjustment is made to normalise for differences in the supporting cell:hair cell ratio in these samples. *Caprin-1* is expressed in supporting cells and hair cells, whereas *Pou4f3* is only in the hair cells. (**B**) Individual hair cells were collected from five different experiments. After determining that samples were hair-cell-specific (using *Myo7a* expression and $p27^{kip1}$ exclusion), *Caprin-1* and *Pou4f3* levels were measured by qPCR. Expression levels were calculated relative to expression of an individual cell at 0 hours to determine the relative quantification (RQ) value. Individual hair cell results are plotted (each symbol represents a different experiment) as well as the mean value for each time point (grey bar). The s.e.m. for each experiment was as follows: *Caprin-1*, 0 hours ±0.98, 3 hours ±1.76, 12 hours ±5.80; *Pou4f3* 0 hours ±0.42, 3 hours ±0.31, 12 hours ±0.90. (**C**) Individual hair cells collected from the culture in Experiment 1 (see A and dark blue triangles in B). Expression levels are calculated relative to the expression at 0 hours and are plotted on a logarithmic scale. All error bars represent the 95% confidence interval.

were seen in the cytoplasm of hair cells. Caprin-1 was also observed in these granular structures, which were located both towards the apical pole (Fig. 5, Upper) and around the nuclei of hair cells (Fig. 5, Lower; supplementary material Fig. S2). In most cases Caprin-1-positive granular structures were also labelled with TIA-1. At 6 hours, the first signs of disruption in hair cell chromatin were observed, suggesting that the cells were undergoing cellular stress. These data provide strong evidence that stress granules form in mammalian hair cells in response to aminoglycoside treatment. They also show, for the first time, that Caprin-1 associates with stress granules in hair cells, indicating that it is a component of the stress response in these cells.

Profiling changes in *Pou4f3* and *Caprin-1* mRNA in the cochlea after ototoxic damage

We used qPCR to determine whether the change in Caprin-1 protein expression in hair cells after neomycin treatment was correlated with the activation of *Caprin-1* transcription. *Caprin-1* mRNA levels were assayed in both mixed-cell samples and single hair cells aspirated from cochlear cultures that had been treated with neomycin for 0, 3 and 12 hours (Fig. 6). Data from the mixed-cell samples indicated the average changes in gene expression from pooled Deiters' and hair cells. Immunofluorescence data (see adjacent cells in Fig. 5, 6 hours) indicated that, at any time point during the antibiotic regime, individual hair cells show different

levels of stress and concomitant change in Caprin-1 expression. Sampling at the single-hair-cell level is likely to document such changes, as well as providing hair-cell-specific data. Further comparison of data from individual hair cells and mixed-cells aspirated from the same cultures should also provide a basis for determining whether any changes in Caprin-1 expression are derived from hair cells or supporting cells.

In the mixed-cell samples, any change in *Caprin-1* mRNA could be influenced by differences in the ratio of hair and supporting cells aspirated in each sample, particularly as *Caprin-1* appears to be expressed at higher levels in supporting cells. The potential for this to influence *Caprin-1* expression data can be seen in Fig. 4D. We therefore normalised the level of *Caprin-1* mRNA to that of the supporting cell marker $p27^{kip1}$ in these samples to give an indication of the effects of neomycin on *Caprin-1* after normalising for the proportion of supporting cells (Fig. 6A). A similar normalisation was made for *Pou4f3* (a hair-cell-specific gene) by using the levels of the hair cell marker *Myo7a* (Fig. 6A). In these mixed-cell samples, we observed increased *Caprin-1* expression in response to neomycin treatment in two out of three experiments. In both these experiments we also observed a drop in *Pou4f3* levels after 3 hours. In a third experiment, where there was an increase in *Pou4f3* levels after 3 hours, there was no increase in *Caprin-1*.

To clarify whether the increase in *Caprin-1* is hair-cell- or supporting-cell-derived, we examined single-cell qPCR data from individual hair cells collected from five independent experiments. Fig. 6B shows that, although there was a degree of variability between individual cells, *Caprin-1* levels in hair cells increased after 3 and 12 hours of neomycin treatment, with a ~17-fold mean induction after 12 hours. Comparison of individual hair cells collected from the cultures used for the mixed-cell samples in Experiment 1 (Fig. 6C) indicated that this increase in hair cell *Caprin-1* was much greater than that observed in the mixed-cell sample from the same culture. This suggests that the induction we observed in that mixed-cell sample is predominantly hair-cell-derived. *Pou4f3* mRNA levels were variable in these individual hair cells, something we routinely observed for *Pou4f3* in single-cell qPCR. However, normalised *Pou4f3* data obtained in the mixed-cell sample in Experiment 1 indicated that *Pou4f3* levels were reduced in hair cells in these cultures (Fig. 6A). To provide a separate estimate of *Caprin-1* changes from a larger cell population, outer-hair-cell-enriched samples were collected from a further ten explant cultures that had been treated with neomycin for 6 hours; their mRNA was pooled and compared with the levels in untreated explant cultures. In these experiments, $p27^{kip1}$-normalised *Caprin-1* mRNA expression was again significantly increased in the neomycin-treated cultures, with an average 1.39-fold increase [*Caprin-1* untreated=1.0 (95% confidence interval: 0.95–1.05); *Caprin-1* with neomycin=1.39 (95% confidence interval: 1.29–1.62)].

Discussion

Caprin-1 is a regulatory target of Pou4f3 and is activated in hair cells during ototoxic damage

We have shown that *Caprin-1* is a target of the sensory hair cell transcription factor Pou4f3, which represses *Caprin-1* expression by binding within a region ~4.7 kb upstream of the mouse *Caprin-1* transcription start site. Our combined ChIP and reporter gene assay results suggest that *Caprin-1* is a direct target of Pou4f3 regulation and that the binding of Pou4f3 to the upstream site A1 is primarily responsible for this regulation. Previously published Pou4f3 targets have all been upregulated, although not all are regulated through direct interaction with the target promoter (Clough et al., 2004; Hertzano et al., 2007; Hertzano et al., 2004). POU domain transcription factors have the ability to act as both repressors and activators of transcription, and can be influenced by interactions with cofactors (Budhram-Mahadeo et al., 1996; Dawson et al., 1996a; Dawson et al., 1996b; Phillips and Luisi, 2000), as well as by the spacing and context of response elements within the target promoter (Remenyi et al., 2002).

To date, Caprin-1 expression has largely been studied in cell lines. Here, examination of Caprin-1 expression at both the mRNA and protein level in the cochlea indicates that Caprin-1 is expressed in native cells, with the level of expression being somewhat lower in sensory hair cells than in the surrounding glial-like supporting cells, such as Deiters' and Claudius' cells. This expression pattern is inversely correlated to the established Pou4f3 expression pattern, suggesting there are lower levels of *Caprin-1* in cells that express Pou4f3 and supporting the idea that Pou4f3 negatively regulates *Caprin-1* in vivo. However, the presence of *Caprin-1* in undamaged hair cells demonstrates that it is not completely repressed by Pou4f3 in these cells and that its expression can be regulated by other mechanisms. Rather, on the basis of our data, a model of the relationship between Pou4f3 and *Caprin-1* is that a damage-induced reduction in, or modification of, hair cell Pou4f3 would lead to the upregulation of *Caprin-1* expression and stress granule formation in hair cells. Experiments in OC-2 cells indicate that a threshold effect exists for the ability of Caprin-1 to trigger stress granule formation, allowing us to speculate that relief of Pou4f3-mediated repression of *Caprin-1* could allow levels to reach this threshold.

To investigate the relationship between Caprin-1 and Pou4f3, we performed the technically difficult task of using qPCR on aspirated single cells and mixed-cell samples, after 0, 3 and 12 hours of neomycin exposure, in order to document changes in hair cell *Pou4f3* and *Caprin-1*. Consistent with the differences between cells in the timing of hair cell damage and the change in Caprin-1 expression observed by immunofluorescence, the qPCR data show differences in *Caprin-1* induction between cultures. In two out of three cultures, we detected an increase in *Caprin-1* mRNA levels, which correlated with a decrease in *Pou4f3*. This increase in *Caprin-1* expression was also observed in individual hair cells. In addition, comparison of single-cell and mixed-cell qPCR in samples collected from the same culture suggested that the increase in Caprin-1 we observed is through activation of its transcription in hair cells. Hence, the relative change observed in individual hair cells was much greater than the increase detected in the mixed-cell sample from the same cultures. Taken together, this suggests that the induction of *Caprin-1* during neomycin treatment was specific to hair cells, consistent with it being mediated by Pou4f3. Documenting consistent changes in *Pou4f3* mRNA levels with single-cell qPCR proved more problematic in these experiments. It is possible that *Pou4f3* mRNA is highly dynamic and that its levels vary greatly between individual cells or, more likely, that the transcript is expressed at such a low level that it cannot be reliably quantified in our single-cell qPCR assay. However, qPCR data performed on mixed-cell samples collected from the same neomycin-treated cultures showed reduced *Pou4f3* mRNA levels that correlated with the induction of *Caprin-1* mRNA at both 3 and 12 hours. *Pou4f3* is specific to hair cells (Xiang et al., 1997) and, hence, we conclude there is a neomycin-induced reduction of *Pou4f3* in hair cells. The present data from OC-2 cells demonstrate

a mechanism for Pou4f3-mediated repression of *Caprin-1* expression, and qPCR data from native tissue show a differential increase in hair cell *Caprin-1* during damage, which correlates with reduced levels of *Pou4f3* in those cultures.

Caprin-1 induces stress granule formation in OC-2 cells and is a component of hair cell stress granules during ototoxic damage

In HeLa and NIH 3T3 cell lines, Caprin-1 has been shown to associate with the cytoplasmic stress granules that form in response to arsenite treatment (Shiina et al., 2005; Solomon et al., 2007). Particular mRNAs are selectively recruited to these granules to regulate their translation, enabling the cell to respond to stress and potentially recover (Kedersha et al., 2000; Kedersha et al., 1999). It has been suggested that, if the duration of mRNA reprogramming by stress granules extends beyond a certain threshold, apoptosis occurs (Anderson and Kedersha, 2002b). Therefore, stress granule formation is considered a key decision point in the response to a number of cellular damage mechanisms. Here, we show that exogenous expression of GFP–Caprin-1 in OC-2 cells induces the formation of mobile dynamic aggregates identified as stress granules, in agreement with previous work in other cell lines (Shiina et al., 2005; Solomon et al., 2007). In addition, we found that mammalian cochlear hair cells undergoing aminoglycoside-induced damage form TIA-1- and Caprin-1-containing granules, indicating that they are stress granules and identifying Caprin-1 as a component of the stress granule response in native tissue for the first time. Whether a similar response is also observed in vivo, in the adult cochlea or following other types of damage to hair cells, such as noise exposure, remains to be established.

Aminoglycoside antibiotics, such as gentamicin and neomycin, are still widely used, despite being ototoxic, and thus remain a common cause of permanent hearing loss (Guthrie, 2008). These drugs selectively accumulate in hair cells, with the primary entry site probably being the hair cell transducer channel (Gale et al., 2001; Marcotti et al., 2005). During aminoglycoside-induced hair cell damage, we hypothesise that Caprin-1 binds specific mRNAs within hair cell granules and regulates their translation, thus contributing to the cell's response to this environmental stress. This stress-granule-based mechanism might offer a window of opportunity for a cell to recover, as long as the insult is temporary or, upon longer-term insult, the same mechanism could promote the controlled removal of a damaged cell by apoptosis (Arimoto et al., 2008). Although they have been predominantly studied in cell lines, stress granules have been identified in an animal model for stroke in neurons following ischaemia (Kayali et al., 2005), as well as in tumour cells after whole-animal radiotherapy (Moeller et al., 2004). The formation of Caprin-1-containing stress granules in cochlear hair cells following a clinically relevant damage paradigm adds to the increasing evidence that stress granules have an important function in situations of cell stress in vivo.

Here, we implicate Pou4f3 in this stress-granule-based response. Mutations in Pou4f3 cause progressive adult-onset hearing loss, presumably due to hair cell death. The identification of Caprin-1 as a negatively regulated Pou4f3 target gene involved in a cellular stress response pathway and, in this case, the death of hair cells, is consistent with the known survival-promoting role of Pou4f3 during the maturation of these cells. Our finding that a Caprin-1–stress-granule pathway is also induced by an environmental agent of hair cell loss links this to both genetic and environmentally induced hearing loss. Previous evidence of a link between POU domain transcription factors and stress responses comes from an RNAi (RNA interference) screen for human genes involved in stress granule assembly, which identified the closely related POU4 family member *POU4F1* as a gene required for stress granule assembly (Ohn et al., 2008). Taken together with our results, this implicates the POU4 family in regulating this form of stress response in multiple systems and here we suggest a mechanism through which this can occur.

In conclusion, our findings indicate that *Caprin-1* is a target that is negatively regulated by Pou4f3, and, in identifying this, we have described for the first time the involvement of the stress granule response pathway in mammalian hair cells exposed to a clinically relevant ototoxic agent. We conclude that Caprin-1 is a stress granule component in native tissue and our results link POU domain transcription factor regulation with a cellular stress pathway that could have relevance for other systems.

Materials and Methods

Cell and organ explant culture

UB/OC-2 cells were cultured at 33°C under 5% CO_2 in Eagle's minimal essential medium containing L-glutamine and supplemented with 10% fetal calf serum (FCS) and 50 units/ml interferon-γ (all Invitrogen). Cochleae were isolated from postnatal day 2 Sprague–Dawley rats (Lahne and Gale, 2008), killed in accordance with the United Kingdom (Scientific Procedures) Act of 1986. Cochlear explants were cultured in glass-bottomed dishes (MatTek) coated with CellTak (BD Biosciences). Explant cultures were incubated in Dulbecco's modified Eagle's medium (DMEM) plus Ham's F12 with L-glutamine (Invitrogen) containing 1% FCS and were maintained at 37°C under 5% CO_2 for at least 16 hours before use in experiments. Explant cultures were incubated with serum-free medium with or without 1 mM neomycin for the time specified. FM1-43 (3 μM) (Gale et al., 2001) was used to aid identification of hair cells during collection, which was from the basal end of the middle turn of the cochlea.

Pou4f3 manipulation in cells

UB/OC-2 cells were manipulated for subtractive hybridisation analysis to identify Pou4f3 target genes. Briefly, cells were transfected with a MacK^KII (Miltenyi Biotech) expression vector containing the mouse Pou4f3 cDNA (+Pou4f3) or the N-terminus of Pou4f3 in the antisense orientation (a/s Pou4f3). At 10 days after transfection, utilising the H-2K^K cell surface marker, which is expressed on the surface of MacK^KII-transfected cells, stably transfected cells were isolated by three rounds of magnetic selection, and returned to culture at low density. After 1 week, plaques of cells derived from a single cell were picked and transferred into individual wells of a 24-well plate. After further culture, cells were screened for expression of the exogenous RNA using reverse transcription-PCR (RT-PCR), cultured further and poly(A)-positive (polyA+) mRNA prepared for subtractive hybridisation.

Subtractive hybridisation

Subtractive hybridisation (Clontech PCR-select cDNA subtraction kit) was performed with 2 μg of polyA+ mRNA, as per the manufacturer's instructions, in both the forward (+Pou4f3 minus a/s Pou4f3) and reverse (a/s Pou4f3 minus +Pou4f3) directions. Subtracted cDNA libraries were constructed by TA cloning into pGemT (Promega) followed by transformation into JM109 *E. coli*; colonies were pricked into 96-well plates [Luria–Bertani medium with ampicillin (LBamp)], grown overnight at 37°C and stored at –80°C as glycerol stocks. Each of these 96-well format clones were then pricked onto four replicate LBamp agar plates [with Hybond N+ membrane (GE Healthcare) on the surface] and incubated at 37°C overnight. The membranes were removed, and the cDNAs were denatured and fixed onto the membrane by alkali fixation, to produce colony arrays. Arrays were screened for differential expression by ^{32}P-labelling the subtracted and non-subtracted cDNAs as per the manufacturer's instructions. This comprised two rounds of colony array, and then an array with the PCR-amplified insert, which was subsequently followed by virtual northern blotting with cDNA from the transiently transfected cells rather than the stably transfected cells. At each screening stage, clones were selected for the next round on the basis of differential expression patterns (Fig. 1A).

Plasmid DNA constructs

The *Caprin-1* luciferase reporter construct (4.7kb-*Cap-1*-LUC) was generated by cloning a 4.7-kb fragment from the mouse *Caprin-1* 5′ flanking sequence into pGL4.10 (Promega). *Cap1*-A-LUC and *Cap1*-C-LUC were made by subcloning 1.2-kb and 840-bp fragments from 4.7kb-*Cap-1*-LUC into pGL4.23 (Promega). Wild-type and ddl Pou4f3 expression vectors have been described previously (Clough et al., 2004). GFP-fused wild-type and truncated Caprin-1 plasmids (for expressing GFP–Caprin-1 and GFP–Caprin-1-606) were kindly provided by John W. Schrader

(University of British Columbia, Vancouver, Canada) and have been described previously (Solomon et al., 2007).

Transient transfections

For dual luciferase assays, OC-2 cells were plated in six-well plates and transfected with 0.2 μg of the reporter construct, various amounts of Pou4f3 expression vector (as specified in Fig. 2) and 10 ng of phRL-null (Promega). The total amount of transfected DNA was kept constant using empty pSi plasmid (Promega). After ~16 hours, cells were glycerol-shocked and cultured for 24–36 hours. Following this, cells were harvested and promoter activity determined using the Dual Luciferase Reporter Assay system (Promega). Each experiment was performed in triplicate, using at least two different DNA preparations. Control experiments verified no effect of Pou4f3 on the pGL4.10 or pGL4.23 plasmid backbone.

For imaging, OC-2 cells were plated onto 13-mm glass coverslips (for immunofluorescence) or onto 50-mm-diameter glass-bottomed dishes (MatTek) (for time-lapse experiments). Cells were transfected with 0.5–1 μg of the GFP–Caprin-1 or GFP–Caprin-1–606 expression vectors using FugeneHD (Roche) (following the manufacturer's protocol at a 5:2 ratio of FugeneHD to DNA).

Quantitative real-time PCR

Brain, cochlea and utricles were isolated from P18 mice and transferred into RNAlater (Qiagen). RNA was extracted using an RNeasy kit (Qiagen) and, following treatment with RQ1 RNase-free DNase (Promega), was reverse-transcribed with Omniscript reverse transcriptase (Qiagen) using random primers (Promega).

For the isolation of different cell types from ex vivo cochlea cultures, cells were aspirated using a glass micropipette from cultures that had been treated with the fluorescent dye FM1-43. This allowed for the selective collection of hair cells (without the use of enzymes), as well as identification and collection of surrounding supporting cell types by their proximity to FM1-43-positive hair cells. Outer-hair-cell-enriched samples contained ~40–50 cells; supporting-cell-enriched and mixed-cell samples contained up to ~250 cells. Cells isolated from ex vivo cochlea cultures were transferred into lysis buffer (provided with the Cells-to-cDNA kit; Ambion) and the cDNA was reverse-transcribed using the Cells-to-cDNA II kit (Ambion). For neomycin-treated single-cell experiments, eight individual cells were collected from each culture for analysis to determine hair cell specificity in five different experiments. Specific targets were preamplified using TaqMan PreAmp Master Mix (Applied Biosystems).

Gene expression was determined by qPCR using gene expression assays from Applied Biosystems: *p27^{kip}* (assay number Rn00582195_m1), *Myo7a* (assay number Rn00596450_m1), *Pou4f3* (assay number Mm00454761_m1) and *Caprin-1* (assay number Rn01512768_m1). Reactions were multiplexed with a primer-limited eukaryotic 18S rRNA endogenous control, performed in triplicate and amplified on a SDS7500 real-time PCR System (Applied Biosystems). Relative quantification studies were performed using SDS1.2.1 software (Applied Biosystems). The few single-cell samples in which *Caprin-1* could not be detected were not included in the analysis.

Electrophoretic mobility-shift assay

Nuclear extracts were prepared from OC-2 cells as previously described (Alkalay et al., 1995). Double-stranded oligonucleotides (Fig. 1B) were labelled with [γ-^{32}P]ATP using T4 kinase. Binding reactions were prepared without labelled oligonucleotide using 8% Ficoll in 20 mM HEPES, pH 7.9, 50 mM KCl, 1 mM EDTA, 0.5 mM DTT, 0.3 μg poly(dI·dC) and ~4–6 μg of nuclear extract from OC-2 cells, to a total volume of 20 μl. For competition assays, 250 ng of unlabelled double-stranded oligonucleotide was also added. This reaction was incubated on ice for 10 minutes, then 1 ng of labelled probe was added and the reaction was incubated for a further 30 minutes at room temperature. Reactions were loaded onto a 4% polyacrylamide 0.25× TBE gel and run at 200 V for 2–3 hours at 4°C, before the gel was dried and exposed to X-ray film.

Chromatin immunoprecipitation

Pou4f3 cDNA was cloned into the pHT2 HaloTag Vector (Promega) and transfected into cells using FugeneHD, as described above. Cells were harvested at 36 hours after transfection and ChIP assays performed in accordance with the Halo-CHIP protocol (Promega). Real-time PCR analysis of recovered DNA was performed using the Power SYBR Green PCR master mix and six sets of primers were assessed (targeting the five putative Pou4f3-binding sites and a nearby negative control region). The levels of precipitation were compared with that of the empty vector and the control region within the *Caprin-1* promoter. Primer sequences are available from the corresponding author on request. Real-time PCR was performed in triplicate and dissociation analysis was performed to ensure specific amplification.

Immunofluorescence and cochlea slicing

Antibodies were against the following proteins: Caprin-1 (rabbit, 1:200) (Solomon et al., 2007); TIA-1 (C-20) (goat polyclonal antibody, 1:200; Santa Cruz Biotechnology); myosin VIIa (138-1) [mouse, 1:250; developed by Soni et al. (Soni et al., 2005) and obtained from the Developmental Studies Hybridoma Bank (Department of Biology, University of Iowa, Iowa City, IA)]. Secondary antibodies (all Invitrogen) were used at 1:1000: goat anti-(rabbit Ig) conjugated to Alexa Fluor 488 or Alexa Fluor 546; goat anti-(mouse Ig) conjugated to Alexa Fluor 568 or Alexa 635; donkey anti-(goat Ig) conjugated to Alexa Fluor 488; and rabbit anti-(goat Ig) conjugated to Alexa Fluor 568. Cells were fixed in 4% paraformaldehyde for 30 minutes, and permeabilised and blocked in 0.5% Triton X-100 with 10% serum for 1–2 hours, before incubation with primary antibody overnight at 4°C. After rinsing, cells were incubated with the secondary antibody for 2 hours. Nuclei were stained with 1 μM DAPI. Samples were imaged on a Zeiss LSM Meta 510 confocal microscope (Zeiss) using 20× (0.8 NA) and 63× (1.4 NA) objectives. In order to provide unbiased sampling of the cochlear coils, the tissue was viewed using bright-field imaging; areas were then selected without observing the antibody fluorescence. Stress granule formation was examined in at least nine individual experiments and representative images are shown in the figures. For cochlear slices, cochleae were obtained from P2 Sprague–Dawley rats and fixed in 4% paraformaldehyde for 60 minutes. After washing, cochleae were mounted in 4% low-melting-point agarose. Slices (300 μm thick) were cut using a 1000 Plus Vibratome (Intracel). Immunofluorescence was performed on slices obtained from three different cochleae.

Time-lapse confocal microscopy

A Hamamatsu Orca-ER cooled charge-coupled device (CCD) camera on an UltraVIEW RS spinning disc confocal scanner (PerkinElmer) was used for live imaging of OC-2 cells within a 37°C and 5% CO_2 chamber. Bright-field and *z*-stack GFP fluorescence images were captured using a 20× objective at 7–10 minute intervals using UltraView software.

We thank John W. Schrader (University of British Cololumbia, Vancouver, Canada) for the supply of anti-Caprin-1 antibody and Caprin-1 expression constructs, Karen Avraham (Sackler School of Medicine, Tel Aviv University, Tel Aviv, Israel) for the supply of ddl cDNA, Daniel Jagger (UCL Ear Institute, London, UK) for help and expertise with cochlear slices, and Lisa Nolan (UCL Ear Institute, London, UK) for comments on the manuscript. This work was primarily funded by a grant from the Royal National Institute for the Deaf [grant number G28 (to J.E.G. and S.D.)] and also by a Royal Society University Research Fellowship (to J.E.G.). Additional experiments were funded by a grant from Deafness Research UK [grant number 483 (to J.E.G. and S.D.)]. The original subtractive hybridisation screen was supported by a grant from the Wellcome Trust [grant number 064599 (to S.D.)]. J.K. is supported through a Deafness Research UK PhD studentship. Deposited in PMC for release after 6 months.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/124/7/1145/DC1

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