Cell Death and Differentiation (2016) 23, 608–615

www.nature.com/cdd

An RNA interference screen identifies new avenues for nephroprotection

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Acute kidney injury is a major public health problem, which is commonly caused by renal ischemia and is associated with a high risk of mortality and long-term disability. Efforts to develop a treatment for this condition have met with very limited success. We used an RNA interference screen to identify genes (*BCL2L14*, *BLOC1S2*, *C2ORF42*, *CPT1A*, *FBP1*, *GCNT3*, *RHOB*, *SCIN*, *TACR1*, and *TNFAIP6*) whose suppression improves survival of kidney epithelial cells in *in vitro* models of oxygen and glucose deprivation. Some of the genes also modulate the toxicity of cisplatin, an anticancer agent whose use is currently limited by nephrotoxicity. Furthermore, pharmacological inhibition of *TACR1* product NK1R was protective in a model of mouse renal ischemia, attesting to the *in vivo* relevance of our findings. These data shed new light on the mechanisms of stress response in mammalian cells, and open new avenues to reduce the morbidity and mortality associated with renal injury.
Cell Death and Differentiation (2016) **23,** 608–615; doi:10.1038/cdd.2015.128; published online 13 November 2015

Acute kidney injury (AKI), also known as acute renal failure, is an abrupt decrease in kidney function characterized by accumulation of creatinine and urea in the blood. It is a global clinical problem with increasing incidence, dire consequences, unsatisfactory therapeutic options, and an enormous financial burden to societies worldwide.[1,2] The reported prevalence of AKI varies from 1% to upward of 25% depending on the diagnostic criteria and the study population, with especially high incidence among the patients of intensive care units.[2,3] The mortality of these patients may approach or even exceed 50%, and those that survive face prolonged hospitalization and significant increases in morbidity.[3]

Renal ischemia is the major cause of AKI[4] and typically develops following a drop in blood flow to the kidney, causing hypoxia and nutrient deprivation within the affected organ. Although most organs compensate for a reduction in oxygenation by increasing local blood flow, the hypoxic kidney does not increase perfusion,[5] likely because blood does not simply provide sustenance, but also serves as a substrate for energy-consuming renal filtration. Instead, hypoxic kidneys influence blood oxygen content by stimulating erythropoiesis via release of erythropoietin.[6] However, this process is relatively slow, making metabolically active kidney cells highly vulnerable to ischemia.

The initial damage from ischemia is further amplified during reperfusion. The ensuing inflammation creates an environment favorable for fibrosis, which, in turn, permanently attenuates renal function.[7] Chronic kidney disease greatly increases the incidence of new episodes of AKI, thus completing the vicious cycle of organ destruction.[8] Patients undergoing cardiac surgery are known to be at a high risk for ischemic AKI,[9] as are the individuals suffering from sepsis,[10] dehydration[11] and many other conditions. Kidney ischemia is also a prominent factor for kidney transplant patients. In fact, the duration of ischemia experienced by the transplanted kidney is a major predictor for transplant success or failure.[12]

The enormous public health significance has attracted considerable effort to the development of strategies to prevent or reduce the damage from ischemic AKI.[7,13] Current treatment options are aimed at controlling known risk factors in highly susceptible populations or at attempting to replace renal function in patients who have lost it due to injury.[14] Despite major advances in our understanding of the pathology of ischemic injury, current therapies are merely supportive. To the best of our knowledge, no available therapy has been definitively proven to effectively avert the damage to an ischemic kidney.[4] This critical deficiency in the arsenal of modern medicine prompted us to seek suitable pharmacologic targets, which could be exploited for such nephroprotection.

We report here the identification of 10 genes, interference with which protects immortalized renal proximal epithelial cells in an *in vitro* model of ischemia. We also report that some of

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Abbreviations: AKI, acute kidney injury; shRNA, short hairpin ribonucleic acid; BCL2L14, B-cell lymphoma 2-like 14; BLOC1S2, Biogenesis of lysosomal organelles complex-1, subunit 2; C2ORF42, Chromosome 2, open-reading frame 42; CPT1A, Carnitine palmitoyltransferase 1A; FBP1, Fructose-1,6-bisphosphatase 1; GCNT3, Glucosaminyl (*N*-acetyl) transferase 3; RHOB, Ras homolog family member B; SCIN, Scinderin; TACR1, Tachykinin receptor 1; TNFAIP6, Tumor necrosis factor, alpha-induced protein 6; NK1R, neurokinin 1 receptor; TAC1, Tachykinin, precursor 1; HIF-1, hypoxia-inducible factor-1; TRAIL, TNF-related apoptosis-inducing ligand; MAPK, Mitogen-activated protein kinases; CD44, cluster of differentiation 44; GTP, guanosine-5′-triphosphate; BCL-G, B-cell lymphoma-G; FAU, Finkel-Biskis-Reilly murine sarcoma virus ubiquitously expressed; PAXIP1, Paired box 1 interacting (with transcription-activation domain) protein 1; ECT2, Epithelial cell transforming 2; RIST1, Regulator of ischemia and stress tolerance 1; RT-PCR, Reverse transcription PCR

Received 18.5.2015; revised 03.8.2015; accepted 20.8.2015; Edited by G Melino; published online 13.11.15

the genes may be involved in the response to other cytotoxic stresses, and that chemical inhibition of the product of one of the genes reduces the extent of ischemic AKI in animals.

Results

Identification of shRNAs that protect kidney epithelial cells in a model of ischemia. Proximal tubule cells are particularly susceptible to ischemia.[15] A very high metabolic rate is required for mediating ion transport, and these cells have a severely limited capacity for anaerobic glycolysis.[15] Importantly, these cells tend to accumulate toxic by-products of incomplete fatty acid oxygenation, and remain hypoxic for an extended period of time, well after reperfusion.[16] Our effort was inspired by the observations that the death of hypoxic kidney cells is an active process, both in the sense of metabolic maladaptations that inflict the initial biochemical insult, and in the sense of mechanisms that recognize the damage and commit cellular pathways to cell death.[7,13,17] This suggests a possibility of nephroprotection via inhibition of certain cellular factors. RNA interference-based genetic approaches are well suited for discovering such factors.[18] We used the non-transformed proximal tubule epithelial cell line, HK-2,[19] in conditions of low oxygen and glucose as an *in vitro* model of AKI. HK-2 cells are commonly used to study the mechanisms of cell death in renal epithelium.[20]

We screened a pooled lentiviral library containing ~80 000 shRNAs targeting ~16 000 human genes for enhanced survival under low-oxygen, low-glucose conditions (designated as 'ischemic' for the purpose of this study), and chose 23 genes for further examination (Supplementary Figure 1; Supplementary Table 1). The tests confirmed that individual interference with 10 of these genes confers robust protection in our *in vitro* model (Figures 1a and d). More than a fivefold increase in the number of remaining cells was achieved by shRNAs that were not a part of the originally screened pool, confirming that the phenomenon reflects the properties of the intended target genes, and not off-target effects of individual shRNAs. The list of genes included: *BCL2L14*, *BLOC1S2*, *C2ORF42*, *CPT1A*, *FBP1*, *GCNT3*, *RHOB*, *SCIN*, *TACR1*, and *TNFAIP6*. We have tested the efficacy of target suppression by several of the protective shRNAs, and have observed a significant reduction in the levels of expression of the respective genes (Supplementary Figure 2). Importantly, using *RHOB*-targeting shRNAs as an example, we documented that the more efficient shRNAs also render better protection (Figure 1b). In order to exclude cell line specificity of our findings, we repeated the assays using another human

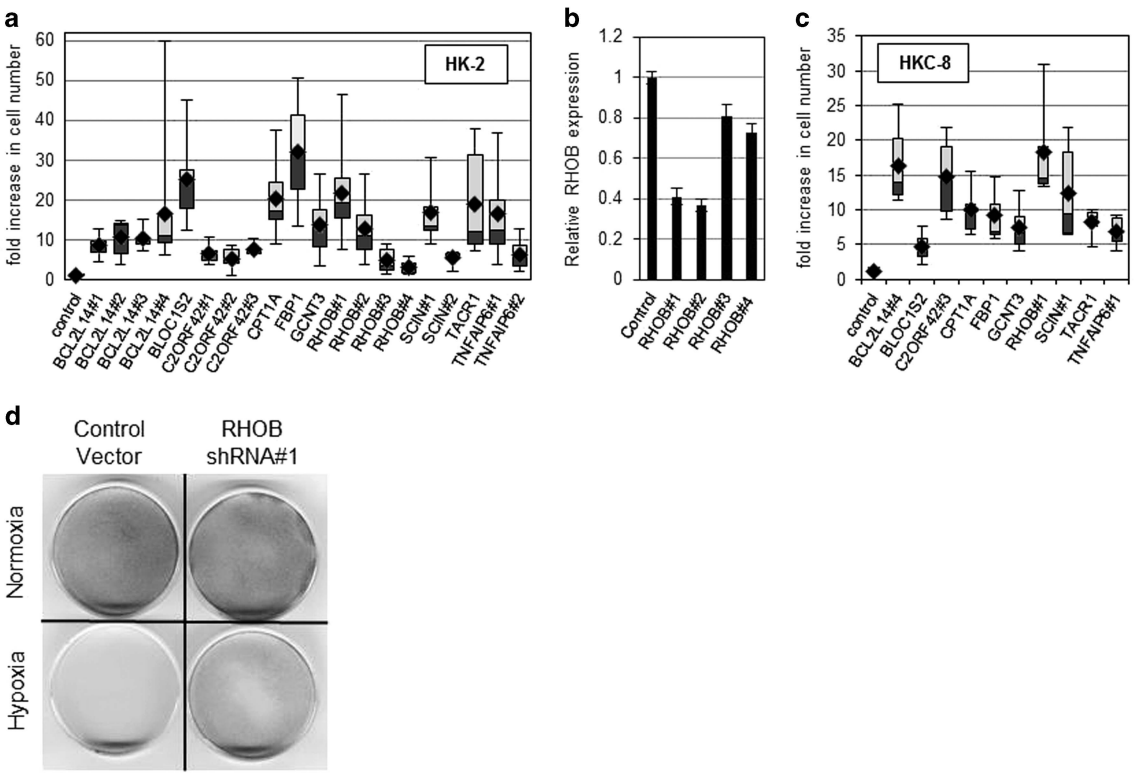

Figure 1 Candidate shRNAs confer resistance to hypoxia in HK-2 cells. (**a**) HK-2 cells expressing candidate shRNAs were subjected to oxygen and glucose deprivation for 48 h. Following treatment, cell numbers for each culture were assessed relative to respective normoxic controls, and values are presented relative to those in parental cells. Cumulative results from at least six independent experiments are shown for each construct. Black rhombuses depict geometric means. Lightly and darkly shaded boxes depict second and third percentiles, respectively. Error bars indicate the ranges. The differences between every individual shRNA and the control vector are significant ($P<0.05$). (**b**) *RHOB* mRNA levels in HK-2 cells transduced with four different *RHOB*-specific shRNAs were measured by qRT-PCR, normalized to mean *GAPDH* transcript levels and reported relative to that in cells transduced with the control vector (pGIPZ). The means and S.D. of three independent experiments are shown. (**c**) HKC-8 cells expressing candidate shRNAs were subjected to oxygen and glucose deprivation for 48 h. The data for three separate experiments were processed and presented as in **a**. The differences between every individual shRNA and the control vector are significant ($P<0.05$). (**d**) Representative images of parallel cultures of HK-2 cells, which were transduced with the control vector or a protective shRNA (RHOB#1). Hypoxia treatment was performed as described in the Materials and methods section, and the remaining cells were visualized by methylene blue staining

renal proximal tubular cell line, HKC-8,[21] with the shRNAs that showed efficacy in HK-2 cells. All 10 tested shRNAs showed robust protection under the selective conditions (Figure 1c).

The role of the identified genes in kidney cell response to cisplatin. In addition to ischemia, AKI can result from exposure to toxins, including various therapeutic agents. For example, renal toxicity of cisplatin limits dose escalation for this anticancer compound, prevents the use of this drug in patients with compromised renal function, and may lead to chronic kidney disease in the treated population.[22] We investigated whether any of the identified genes may affect the response of kidney epithelial cells to cisplatin. Interference with *BCL2L14*, *RHOB* and *C2ORF42* provided robust protection in HK-2 and HKC-8 cells (Figures 2a and b), whereas interference with *TNFAIP6* yields very strong protection in HKC-8 cells only (Figure 2b), indicating that the mechanisms of drug response are not identical between the cell lines. This is also evident from the difference in cisplatin tolerance between the two parental cell lines (Figures 2a and b).

Although normal kidney epithelium is highly sensitive to cisplatin, clear-cell renal cell carcinoma is notoriously unresponsive to conventional chemotherapy. Interestingly, this remarkable change from drug sensitivity to resistance during tumor evolution is paralleled by the loss of *C2ORF42* expression (Figures 2c and d).[23,24]

Protective effects of NK1R inhibition *in vitro* and *in vivo*. Among the identified genes, the product of *TACR1* (neurokinin 1 receptor, NK1R) is inhibited by a number of well-characterized drugs, some of which are used clinically. We hypothesized that those compounds would recapitulate the effects of *TACR1* shRNA on ischemia tolerance. Indeed, NK1R antagonists, L-733,060 and Aprepitant, conferred resistance to ischemia in both HK-2 and HKC-8 cells (Figures 3a and d) at concentrations that had minimal effect on the survival of normoxic cells (Supplementary Figures 3a and d). Furthermore, significant protection was achieved by knockdown of *TAC1*, which encodes for a known NK1R ligand, substance P (Supplementary Figure 4a).

We further evaluated the consequences of NK1R inhibition in an *in vivo* model of acute renal ischemia. Mice pretreated with 30 mg/kg or 90 mg/kg of L-733,060 or the respective vehicle were subjected to transient ischemia on both kidneys. After 24 h of reperfusion, measurement of blood-based markers of AKI revealed a dose-dependent protection by NK1R inhibition (Figure 4). Levels of creatinine and blood urea nitrogen were significantly ($P<0.05$, Mann–Whitney test) reduced by either dose of the compound (Figures 4a and b), whereas the levels of neutrophil gelatinase-associated lipocalin and osteopontin were significantly reduced by a higher dose ($P<0.05$). The same trend appeared at the lower dose, albeit without reaching statistical significance (Figures 4c and d).

Interference with *RHOB* and *C2ORF42* increases the anti-apoptotic effect of Aprepitant. It is clear that multiple processes contribute to cell death during AKI, and simultaneous targeting of multiple pathways may be needed for maximal protection.[13] To this end, we investigated whether interference with any of the confirmed mediators of ischemic death could further improve the peak protection, which is rendered by chemical inhibition of NK1R. Indeed, we observed that interference with *C2ORF42* or *RHOB* consistently increased the maximal efficacy of the drugs (Figures 5a and b; Supplementary Figure 5).

Apoptosis is reported to be the predominant mode of cell death in response to ischemia.[15] Accordingly, we observed that the degree of protection from ischemia correlates well with a reduction in caspase activity in these treatment groups (Figure 5c).

Discussion

A decade ago it was argued that an effective approach to limit or prevent ischemic renal injury in humans remains elusive, primarily because of an incomplete understanding of the mechanisms of cellular injury.[25] Although molecular nephrology continues to progress, mechanisms that may protect ischemic tubular epithelial cells remain poorly understood. The matter is complicated by the fact that the known cell programs, which improve survival in hypoxia (e.g., the hypoxia-inducible factor-1 (HIF-1)-mediated switch from oxidative phosphorylation toward glycolysis) or hypoglycemia (e.g., the switch from glucose to lipid oxidation), become maladaptive when these conditions coincide. Indeed, HIF-1 may also become pro-apoptotic,[26,27] and the toxic products of partial lipid metabolism contribute to ischemic injury.[28]

It is known that reperfusion amplifies the initial injury. This secondary damage ensues from inflammation, and the release of molecules such as osteopontin by injured cells may serve to attract the immune system.[29] Subsequent fibrosis may preserve overall organ integrity, but diminish its function. Again, HIF-1 activity may be maladaptive at this stage, contributing to inflammation, tubular atrophy, and fibrosis.[30] Importantly, it is likely that avoiding the initial damage to epithelial cells would also prevent damage following reperfusion. An additional reason for targeting primary rather than secondary damage in renal ischemia is that mechanisms of ischemic cell death appear conserved, whereas the particulars of reperfusion injury and the modes of its aversion differ between species[16] and even within species,[31] which limits inferences from animal models.

The recognition of large cohorts of individuals at risk for AKI stimulates the push for treatments that would prevent the injury, rather than mitigate its consequences. Multiple promising strategies, too numerous to be listed here, are aimed at averting AKI by rational metabolic or immune interventions, but have proven to be ineffective or unacceptably toxic in preclinical models and clinical trials.[13,32] Undoubtedly, many new approaches are still at various stages of pre-clinical development, but we are unaware of any of them that have successfully crossed the threshold of clinical testing. Similarly, a large number of targeted strategies intended to mitigate the consequence of ischemia in transplant patients have yielded controversial or negative results.[30]

Of note, the earlier attempts at nephroprotection focused on a handful of molecules and pathways, which have been extensively characterized in prior research. In contrast, we undertook an unbiased approach, as we screened a

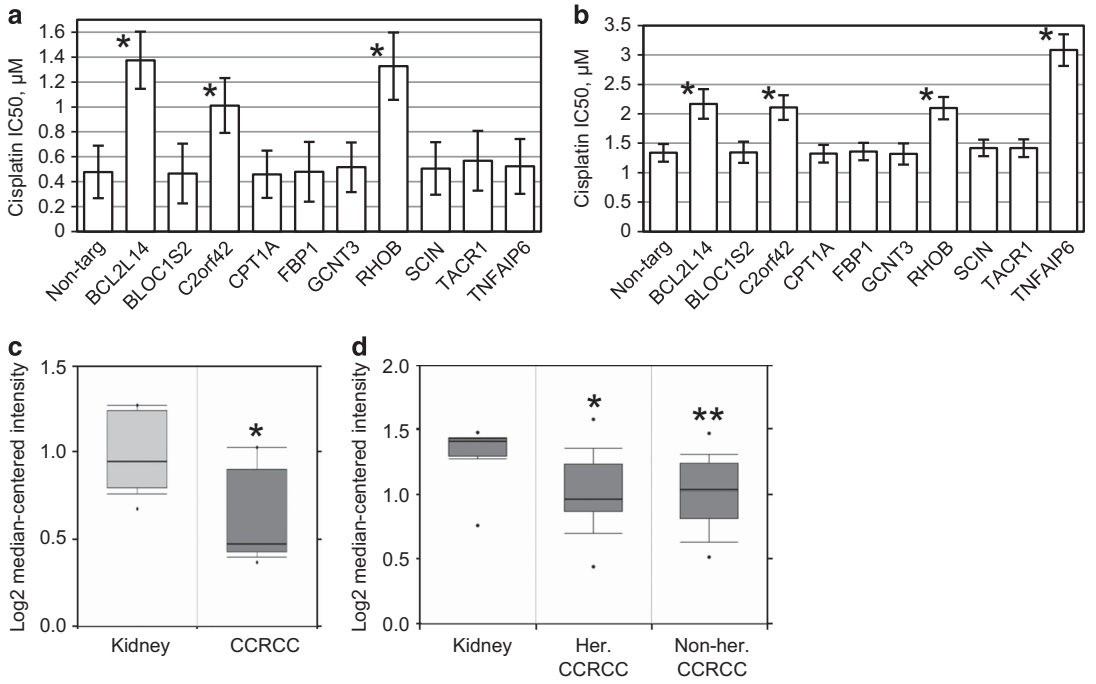

Figure 2 Effects of candidate shRNAs expression on cisplatin-induced cytotoxicity. HK-2 cells (**a**) and HKC-8 cells (**b**), which harbored a control non-targeting shRNA or the shRNAs against the indicated genes, were treated for 48 h with cisplatin concentrations ranging from 0 to 25.6 μM, followed by 3 days in drug-free medium. For each culture, numbers of remaining cells were compared with those in respective untreated controls using methylene blue staining and the extraction method. The bars represent the IC_{50} values. The error bars depict 95% confidence intervals. Asterisks indicate a significant ($P<0.05$) increase in IC_{50} over the respective controls. (**c**) C2ORF42 expression is commonly decreased in clear-cell renal cell carcinoma (CCRCC). The data were retrieved from www.oncomine.com., $^*P=0.001$. (**d**) C2ORF42 is decreased in both hereditary (her.) and non-hereditary (non-her.) CCRCC. $^*P=1.41E-4$, $^{**}P=2.28E-4$. The data were retrieved from www.oncomine.com

genome-wide shRNA library. Screening such a complex library is vulnerable to stochastic experimental 'noise' and various well-recognized artifacts of shRNA technology.[18,33] Thus, it is imperative to validate any candidate genes by additional tests using either individual shRNAs or alternative approaches, for example, chemical inhibitors. We have validated the effect for 10 out of 23 candidate genes. We cannot conclude that the 13 non-validated genes are irrelevant to ischemic response: the negative results could have ensued from insufficient efficacy of the particular shRNAs, or from adverse off-target effects of these shRNAs that outweighed the protective action. Also, our stringent threshold (fivefold increase in cell number over unprotected control) might have excluded relevant genes with weaker effects. In addition, many relevant genes may have been missed during the initial screening, for example, due to insufficient potency or off-target toxicity of the corresponding shRNAs. Furthermore, the discovery of genes, whose products may be nephroprotective upon upregulation, would require an entirely different set of tools. Nevertheless, the discovery of the 10 modulators of nephrotoxicity provides a starting point for elucidating the relevant pathways using conventional biochemical and genetic approaches.

The identified modulators of nephrotoxicity differ in the biochemical properties of their products and in how well they have been characterized. To further complicate matters, information about their function comes from different cell systems, whereas the specifics of hypoxic cell death may differ even between closely related cell types.[34]

Among these genes, *TACR1* is notable due to the availability of clinically useful inhibitors of its product. Although much more work is needed to confirm clinical utility of these agents against AKI, our *in vivo* observations are encouraging. These results also indicate that our *in vitro* system is capable of producing information relevant to renal ischemia in a whole-organism context. Inhibition of NK1R has been proposed as an avenue of ischemia protection for brain and heart,[35,36] but was viewed as a way of preventing secondary damage from edema and other complications of reperfusion. In contrast, our *in vitro* findings demonstrate that this intervention, at least in the context of kidney epithelial cells, prevents primary damage in the absence of additional cell types. Accordingly, a combination of NK1R and its ligand was reported to kill human neurons and embryonic kidney cells.[37] Unlike our system (Figure 5c), the reported mode of death was non-apoptotic.[37] However, the choice between apoptotic and non-apoptotic programs may be decided by the availability of various cellular factors after the cell has committed to dying.[38]

Although the precise nature of the death-promoting ligand has yet to be elucidated, the fact that HK-2 cells are protected by an shRNA targeting *TAC1* (Supplementary Figure 4a) suggests that a product of that gene (e.g., substance P) might be involved in triggering cell death. A relatively small protective effect of *TAC1* knockdown could be explained by insufficient potency of the shRNA, by the presence of cells in which the shRNA expression construct is aberrant or poorly expressed (such cells would still secrete substance P and affect their neighbors), or by the existence of other pro-apoptotic NK1R

ligands. Interestingly, the naked mole rat, which is adapted to hypoxic environments, has extremely low levels of substance P.[39]

Intriguingly, we observed that interference with *TACR1* affects *GCNT3* expression (Supplementary Figure 4b). As *GCNT3* inhibition is also protective, it is possible that *TACR1*-dependent induction of GCNT3 contributes to ischemic cell death. Importantly, *GCNT3*-deficient mice are viable and fertile, despite some defects in immune functions.[40] Thus, transient inhibition of *GCNT3* is likely to be well tolerated.

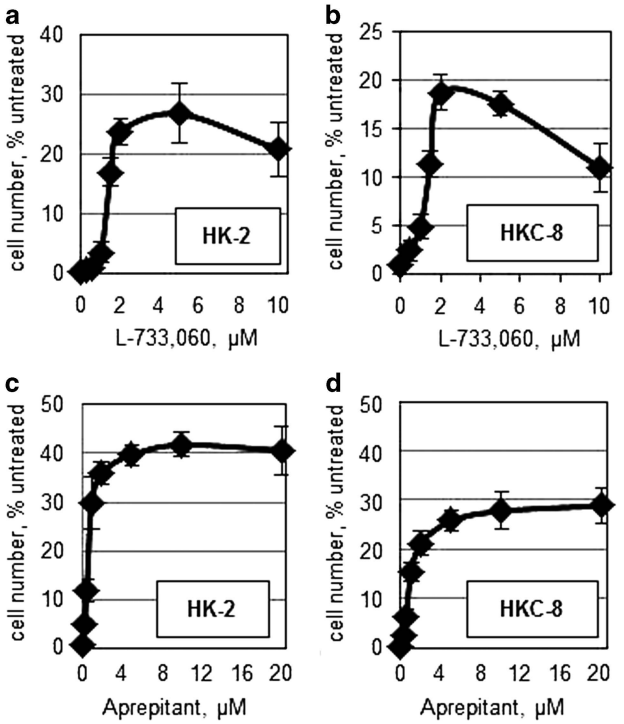

Figure 3 The effects of the NK1R antagonists on hypoxia resistance in kidney epithelial cells. (**a** and **b**) HK-2 (**a**) and HKC-8 (**b**) were treated with L-733,060 and exposed to ischemic conditions for 48 h. For each dose of the drug, the number of cells remaining after treatment was graphed as a percent of cells kept under normoxic conditions. The experiemnts were conducted in triplicate. The error bars depict S.D. (**c** and **d**) HK-2 cells (**c**) and HKC-8 cells (**d**) were treated with Aprepitant and exposed to ischemic conditions for 48 h. The data were processed and presented as in **a** and **b**. In both cell lines, significant ($P<0.05$) protection was observed at concentrations of 1μM and above for L-733,060 and 0.25μM and above for Aprepitant

At this time the mechanism of involvement of TACR1 and GCNT3 in ischemic cell death is unknown. TNF-related apoptosis-inducing ligand (TRAIL) expression is de-repressed by hypoxia, at least, in some cells,[41] and there is evidence that cell death in an ischemic kidney depends on TRAIL signaling.[42] We can also speculate that *N*-acetylglucosaminyltransferase GCNT3 contributes to the glycosylation of TRAIL receptors, which is critical for their function.[43,44] In addition, substance P stimulates p21-activated kinase,[45] which is a known regulator of the mitogen-activated protein kinases (MAPK) cascade.[46] In turn, MAPKs, at least in some cases, may sensitize cells to TRAIL-induced apoptosis.[47] We believe that the hypothetical connection between TACR1 and TRAIL-mediated cell death is worth exploring, because the likely intermediaries include multiple drug-able proteins.

Another gene identified in our study, *FBP1*, encodes a metabolic enzyme, inhibition of which may lead to more efficient utilization of limited resources.[48] A recently reported role of FBP1 in the control of gene expression[49] may also have a role in this process.

CPT1A also encodes a metabolic enzyme. Its product contributes to fatty acid oxidation, and its inhibition may be 'decreasing the oxygen cost of adenosine triphosphate production',[50] as well as preventing the accumulation of toxic, partially oxidized products. Manipulation of CPT enzymes has been considered as a potential therapeutic strategy for treating various diseases, but this is complicated by distinct roles of CPT isoforms as well as the questionable specificity of available inhibitors.[51]

The product of the *BLOC1S2* gene was first described as a protein with a role in biogenesis of lysosome-related organelles, but later research suggested that it is needed for efficient execution of apoptosis in glioblastoma cells.[52]

SCIN encodes a calcium-dependent actin filament-severing protein known to have a role in exocytosis.[53] It acts as a pro-apoptotic protein in megakaryocyte leukemia.[54]

TNFAIP6 is inducible by proinflammatory cytokines, such as tumor necrosis factor-α,[55] which is a known sensitizer to AKI

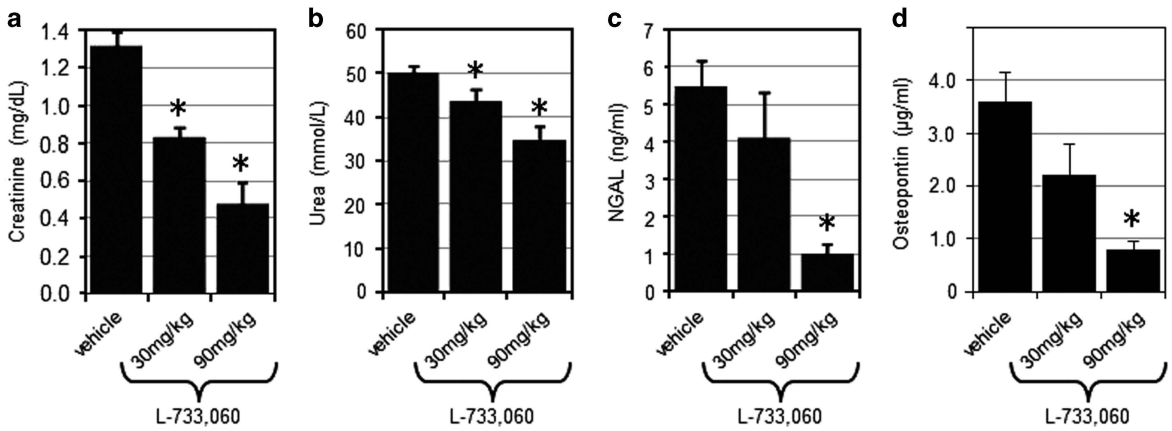

Figure 4 The effects of NK1R inhibitor in a mouse model of kidney ischemia. At 30 min after treatment with the indicated doses of L-733,060, the mice were subjected to 25 min of bilateral renal ischemia. At 24 h following reperfusion, serum levels of creatinine (**a**), urea (**b**), neutrophil gelatinase-associated lipocalin (NGAL) (**c**), and osteopontin (**d**) were measured. The means with S.D. are shown. The asterisks indicate a significant ($P<0.05$) difference from the vehicle-treated group

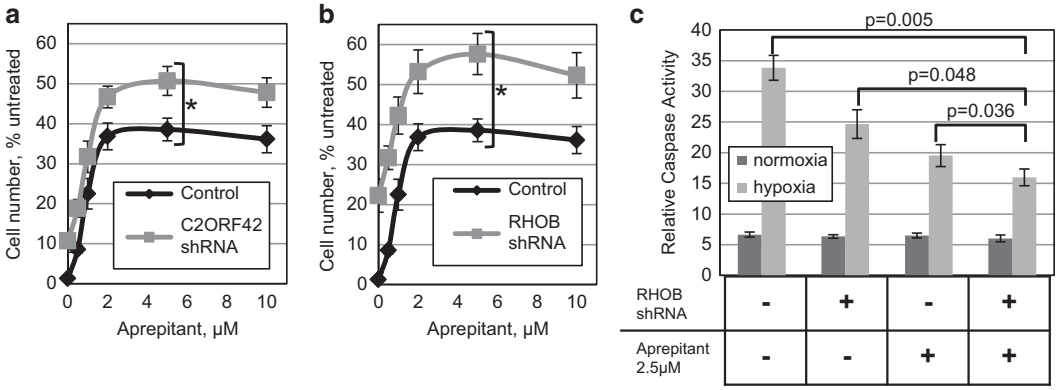

Figure 5 The combination of Aprepitant and select shRNAs enhances hypoxia tolerance in kidney epithelial cells. HK-2 cells expressing shRNA targeting (**a**) *C2ORF42* and (**b**) *RHOB* were treated with the indicated concentrations of Aprepitant and exposed to ischemic conditions for 48 h. For each dose of the drug, the numbers of cells remaining after treatment were analyzed as a percent of cells incubated under normoxic conditions. For each shRNA, the results are shown in comparison with those for vector-transduced HK-2 cells, which were treated in parallel. (**c**) Caspase activity following 40 h of ischemia was measured in HK-2 cells, modified or treated as indicated. Error bars for all panels indicate S.D. of three independent experiments. The asterisks indicate a significant ($P<0.05$, unless otherwise indicated in panel **c**) difference comparing the indicated treatment goups

and a possible target for nephroprotection.[56] Among other roles, *TNFAIP6* modulates the interaction between hyaluronan and CD44,[57] both of which are proposed targets for nephroprotection.[58,59] Importantly, interference with TNFAIP6 reduces epithelial-to-mesenchymal transition in kidney epithelial cells.[60] In conjunction with our findings, this suggests that anti-TNFAIP6 therapy might prevent both the initial damage during ischemia and post-reperfusion fibrosis.

RhoB is a member of the Rho GTP-binding protein family. Its downregulation has been implicated in resistance of some cancer cells to cisplatin.[61] RhoB is activated by a variety of stress signals.[62] In particular, RhoB is activated within minutes of hypoxia in a glioblastoma cell line,[63] and differential regulation of the *RHOB* gene was implicated as a mechanism of uncommon tolerance to hypoxia in a subterranean rodent.[64] Intriguingly, RhoB controls intracellular trafficking,[62] a process that is reportedly affected by other genes identified in our screen (*BCL2L14*, *SCIN* and *BLOC1S2*), and failure to traffic respective receptors is a mechanism of resistance to death-promoting cytokines, including TRAIL.[65,66]

B-cell lymphoma-G (Bcl-G), encoded by *BCL2L14* gene, has been characterized as a pro-apoptotic Bcl-2 family member.[67] This model has been challenged recently by the observation that *BCL214*-deficient dendritic cells are sensitive to apoptosis.[68] Although this might reflect different roles for this protein in different cells and in response to different stressors, there are clues linking Bcl-G with vesicle transport.[68,69] Of note, Bcl-G regulator FAU has been under intense selection in hypoxia-adapted mole rats.[70]

C2ORF42 is the least studied gene on our list. It is conserved between mammals and arthropods. Its product reportedly associates with a mediator of the cell stress response, PAXIP1, and a Rho GTPase regulator, epithelial cell transforming 2 (ECT2),[71] but its biochemical function is unknown. Considering our findings, we propose to name the gene Regulator of Ischemia and Stress Tolerance 1 (RIST1).

In addition to ischemia, exposure to toxins is an important cause of AKI. Nephrotoxicity remains a major side effect of many pharmaceuticals, and patients with chronic kidney disease are especially at risk. Cisplatin is an example of a highly effective drug, for which nephrotoxicity is dose limiting.[72] The status of at least three of the identified genes (*RHOB*, *BCL2L14*, and *RIST1*) strongly affects the response of renal epithelial cell lines to this drug. RhoB has already been implicated in response to DNA damage in other contexts,[61] whereas Bcl-G may be a mediator of the drug-sensitizing functions of its regulator, FAU.[73] Intriguingly, the alleged RIST1-interacting partner, ECT2, also activates RhoB following DNA damage,[74] which may explain similar outcomes of targeting *RIST1* and *RHOB* in our assays. The hypothesis that *RIST1*, *RHOB* and *ECT2* belong to the same biological pathway is further supported by the fact that interference with *ECT2* has an ischemia-protective effect similar to that of *RHOB* and *RIST1* (Supplementary Figure 6). Overall, our findings encourage inquiries into whether interfering with *RHOB*, *BCL2L14*, and *RIST1* and their partners could protect against cisplatin-induced nephrotoxicity without compromising the anti-tumor effect of this drug, and whether these genes have a wider role in on- and off-target toxicity of various pharmaceuticals.

As resistance to hypoxia is a key step in cancer evolution, the mediators of ischemic cell death reported here may have a role in tumorigenesis and tumor resistance to therapy. For example, the common reduction in *RIST1* expression in renal cancer (Figures 2c and d) might offer an explanation for the remarkable resistance of this disease to conventional chemotherapy. Also, the loss of FBP1 has been recently established as an oncogenic event in renal cancer.[49] However, a possible involvement of these genes in cancer is not necessarily incompatible with the development of strategies for nephroprotection based on their inhibition. First, any protective mechanism may depend on the state of metabolism, cell proliferation, and other traits that distinguish a normal cell from a cancerous one. Indeed, *TACR1* inhibitors were discussed as possible anticancer agents.[75] Second, it has been argued that death signaling is commonly impaired in malignancies, so protection of normal cells through targeting of these same pathways may not affect the efficacy of

treatment, at least, for some cancers.[76] Third, an acute nephroprotective intervention may be too short to sustain tumor growth. In fact, there are examples of experimental drugs that protect normal cells from temporary stresses by inhibiting tumor suppressors without long-term oncogenic consequences.[77] Similar approaches for nephroprotection have yielded promising early results.[78]

The differential effects on cisplatin resistance, as well as the differences in the ability to enhance the effect of NK1R inhibitors argue that the identified genes affect multiple pathways of cell death. In addition to achieving maximal protection by a combination therapy, an ability to influence the outcome through several distinct mechanisms provides more options to balance benefits and risks for a given patient. These considerations support further investigation of multiple pathways, elements of which have been uncovered in the current study.

Materials and Methods

Cell culture and viral transduction. HK-2 cells[19] were grown in keratinocyte serum-free medium (Life Technologies, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA, USA), 5 ng/ml epidermal growth factor (Life Technologies), and 100 U/ml penicillin/streptomycin (Life Technologies). HKC-8[21] were maintained in Dulbecco's modified Eagle's medium (Life Technologies) supplemented with 10% fetal bovine serum and 100 U/ml penicillin/streptomycin. Viral transduction was performed as described.[79]

***In vitro* hypoxia treatment.** The cells were subjected to low-glucose medium (Life Technologies) and 0.2% hypoxia for 48 h in a Biospherix Xvivo system (Lacona, NY, USA). Following treatment, the cells were fixed, their numbers were assessed using methylene blue staining and the extraction method,[80] and the values were calculated as percentage of cell number in parallel normoxic cultures. The values for the cultures transduced with various constructs were normalized for the values corresponding to the parental cells. The individual shRNA sequences and their relative effects on HK-2 cells are shown in Supplementary Figure 2.

Cisplatin treatment. Kidney epithelial cells expressing candidate shRNAs and the respective controls were exposed to cisplatin (Sigma-Aldrich, St. Louis, MO, USA) at various concentrations (0, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8, and 25.6 μM) in triplicates for 48 h. Subsequently, the cells were washed and cultured in fresh medium for an additional 3 days. At that time, the cells were fixed, and their numbers were assessed using methylene blue staining and the extraction method.[80] The values were calculated as percentage of untreated cell number, and the IC_{50} values were computed using GraphPad Prizm 6 (GraphPad Software, Inc., La Jolla, CA, USA).

***In vitro* treatment with NK1R antagonists.** The cells were treated with various concentrations of L-733,060 (Tocris, Bristol, UK) and Aprepitant (Selleck Chemicals, Houston, TX, USA) for 1 h before being subjected to hypoxia treatment as described above.

Quantitative RT-PCR. The transcripts were detected using quantitative real-time RT-PCR using *GAPDH* as an endogenous control. The nucleotide sequences were: *RHOB* 5′-ACATTGAGGTGGACGGCAAGCA-3′ and 5′-CTGTCCACCGAGAA GCACATGA-3′; *BCL2L14* 5′-GTAACTGAGGGTCTCTCCTTCC-3′ and 5′-GGAATG GGGATGAAGGCAGTGT-3′; *SCIN* 5′-GCAGAGTATGTAGCAAGTGTCCT-3′ and 5′-GTAAAGCCGAGGTGGATGGTCT-3′; *TACR1* 5′-GCCTGTTCTACTGGAAGTC CAC-3 and 5′-CACAGATGACCACTTTGGTGGC-3′; *GCNT3* 5′-CACCAGAGACTGT GAGCACTTC-3′ and 5′-CATACACAGCTCGCAGTAGCCT-3′ *ECT2* 5′-GCAGTCAG CAAGGTGGCAAGTT-3′ and 5′-CTCTGGTGCAAGGATAGGTCCA-3′; *GAPDH* 5′-GTCTCCTCTGACTTCAACGCG-3′ and 5′-ACCACCCTGTTGCTGTAGCAA. RNA was isolated using the RNeasy, RT kit (Qiagen, Germantown, MD, USA). Complementary DNA was synthesized using SuperScript III (Life Technologies). PCRs were performed using an ABI Prism 7900 Sequence Detection System (Waltham, MA, USA) and IQ SYBR Green SuperMix (Bio-Rad, Hercules, CA, USA). The thermal cycling conditions comprised 2 min at 50 °C, 10 min at 90 °C, and 1 min at 60 °C for 40 cycles. Data were analyzed using RQ Manager 1.2.1 (ABI).

***In vivo* model of ischemia.** The work was approved by the Committee for Animal Experiments of Basel (TierversuchsKommission von BaselStadt). C57BL/6 mice were pretreated with various doses of *TACR1* inhibitor or the corresponding vehicle. After 30 min, mice were anesthetized (with ketamine/rompun). Both kidneys were exposed through flank incisions and kidney pedicles were clamped with atraumatic clamps while the mice were kept at 36 °C. The clamps were removed after 25 min, and the wounds were closed in two layers. After 24 h, blood samples were assayed for the markers of kidney injury.

Caspase activity assay. Apo-ONE Homogeneous Caspase-3/7 Assay (Promega, Madison, WI, USA) was applied to HK-2 cell lysates as per the manufacturer's protocol.

Conflict of Interest

BS, SG, EW, GDN and ME are employees of F. Hoffmann-La Roche AG. The remaining authors declare no conflict of interest.

Acknowledgements. The study was supported in part by the Roswell Park Alliance Foundation. We thank Dr A Chenchik and Dr M Makhanov for their assistance in analyzing the results of the shRNA screening, and Dr W Burhans for his help in preparing the manuscript. This study was supported in part by funds from F. Hoffmann-La Roche AG.

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Supplementary Information accompanies this paper on Cell Death and Differentiation website (http://www.nature.com/cdd)