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Excessive HDAC activation is critical for neurodegeneration in the *rd1* mouse

J Sancho-Pelluz^{1,2}, MV Alavi^{3,4}, A Sahaboglu¹, S Kustermann¹, P Farinelli⁴, S Azadi⁵, T van Veen^{1,4}, FJ Romero², F Paquet-Durand^{*,1} and P Ekström⁴

Inherited retinal degenerations, collectively termed retinitis pigmentosa (RP), constitute one of the leading causes of blindness in the developed world. RP is at present untreatable and the underlying neurodegenerative mechanisms are unknown, even though the genetic causes are often established. Acetylation and deacetylation of histones, carried out by histone acetyltransferases (HATs) and histone deacetylases (HDACs), respectively, affects cellular division, differentiation, death and survival. We found acetylation of histones and probably other proteins to be dramatically reduced in degenerating photoreceptors in the *rd1* human homologous mouse model for RP. Using a custom developed *in situ* HDAC activity assay, we show that overactivation of HDAC classes I/II temporally precedes photoreceptor degeneration. Moreover, pharmacological inhibition of HDACs I/II activity in *rd1* organotypic retinal explants decreased activity of poly-ADP-ribose-polymerase and strongly reduced photoreceptor cell death. These findings highlight the importance of protein acetylation for photoreceptor cell death and survival and propose certain HDAC classes as novel targets for the pharmacological intervention in RP.

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Retinitis pigmentosa (RP) is a group of inherited neurodegenerative diseases that result in selective cell death of retinal photoreceptors. Usually the mutations first lead to degeneration of the rod photoreceptor cells followed by a mutationindependent secondary degeneration of cone photoreceptors. With a prevalence of about 1:3500, RP is considered as the main cause of blindness among the working age population in the developed world. At least 40 RP causing mutations have been identified so far, but the metabolic pathways leading to photoreceptor cell death have not been resolved, and no adequate RP treatment is available.¹

The *rd1* mouse is one of the most studied human homologous RP animal models and carries a loss-of-function mutation in the gene encoding for the β -subunit of rod photoreceptor cGMP phosphodiesterase-6.² This leads to an accumulation of cGMP, which eventually causes photo-receptor cell death.^{3,4} In microarray experiments, we and others found *rd1* degeneration to be accompanied by extensive changes in gene expression.^{5–7} Although some changes may result from direct and specific effects of cGMP

on defined genes,⁸ it is likely that also more generalized alterations of the transcriptional machinery are involved. Previously, we have shown that *rd1* photoreceptor degeneration is in part caused by a strong activation of poly-ADP-ribose-polymerase (PARP),⁹ which may have a bearing on transcriptional activity.¹⁰ However, as PARP activity was found to occur only relatively late during *rd1* degeneration, we hypothesized that there might be yet other mechanisms causing dysregulation of gene expression.

Gene regulation is to a large extent governed by epigenetic mechanisms, among which acetylation of histones¹¹ appears to be one of the most important.¹² Histone acetylation and deacetylation is mediated by histone acetyltransferases (HATs) and histone deacetylases (HDACs), respectively.¹³ The HDAC family is subdivided into three main classes (HDAC I, II, and III), depending on their similarity with homologous yeast genes. Class I (HDAC 1–3 and 8) and class II (HDAC 4–7, 9, and 10) are inhibited by trichostatin A (TSA).¹³ Class III HDACs, also referred to as sirtuins (isoforms: sirt1–7) form a structurally distinct class of

¹Division of Experimental Ophthalmology, Institute for Ophthalmic Research, University of Tübingen, Tübingen, Germany; ²Fundación Oftalmológica del Mediterráneo, Universidad Cardenal Herrera-CEU, Valencia, Spain; ³Molecular Genetics Laboratory, Institute for Ophthalmic Research, University of Tübingen, Tübingen, Germany; ⁴Department of Ophthalmology, Clinical Sciences, Lund, University of Lund, Sweden and ⁵Department of Biochemistry and Molecular Biology, University of British Columbia, BC, Canada

^{*}Corresponding author: F Paquet-Durand, Institute for Ophthalmic Research, University Eye Clinic Tübingen, Röntgenweg 11, Tübingen 72076, Germany.

Tel: + + 49 (0) 7071 29 87430; Fax: + + 49 (0) 7071 29 5777; E-mail: francois.paquet-durand@klinikum.uni-tuebingen.de

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Abbreviations: ab, antibody; abs, antibodies; CBP, CREB-binding-protein; CREB, cyclic AMP-response-element-binding; CRX, cone-rod-homeobox; GCL, ganglion cell layer; HDAC, histone deacetylase; INL, inner nuclear layer; NAD⁺, nicotinamide dinucleotide; NAM, nicotinamide; ONL, outer nuclear layer; P, post-natal day; PFA, paraformaldehyde; PBS, phosphate buffered saline; PDE6, phosphodiesterase-6; PAR, poly-ADP-ribose; PARP, poly-ADP-ribose-polymerase; RP, retinitis pigmentosa; SEM, standard error of the mean; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labelling; TSA, trichostatin A; WB, western blot Received 23.12.09; accepted 04.1.10; Edited by A Verkhratsky

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 $\rm NAD^+$ -dependent enzymes that can be inhibited by nicotina-mide (NAM).^{14}

Although a number of studies have related transcription of photoreceptor genes and photoreceptor viability with histone acetylation,^{15–17} information regarding whether and how HDAC activity connects to degenerating photoreceptors is lacking. Here, we analyzed enzymatic activities of different HDAC classes *in situ* on retinal tissue sections and studied how various inhibitors affect retinal cell viability. Activity of HDACs I/II was strongly elevated in *rd1* photoreceptors and causally related to their death, suggesting HDAC inhibition as a novel approach for neuroprotection in retinal degeneration.

Results

Expression of HDACs in *wt* and *rd1* retina. Microarray analysis of the expression of 13 different HDAC genes did not identify any significant differences between wild type (*wt*)



Figure 1 Micro-array analysis and immunodetection of different HDAC isoforms in *wt* and *rd1* retinae at P11 *in vivo*. mRNA expression for 14 different HDAC isoforms pertaining to all four HDAC classes was assessed using micro-array analysis. (a) For all studied HDAC isoforms, the ratio of mRNA expression *wt/rd1* was not significantly different from 1, indicating that expression was not changed. Values are mean ± SEM from five independent hybridization experiments, each containing retinae from four male *wt* and four *rd1* animals. Immunostaining was performed for three different HDAC isoforms representing HDAC classes I–III. HDAC2 (**b**, **e**) and HDAC5 (**c**, **f**) were expressed in the nuclei of ONL, INL, and GCL, while Sirt2 (**d**, **g**) was expressed in photoreceptor segments and neuritic processes in the ONL and different INL cell types. No obvious differences between *wt* (**b-d**) and *rd1* (**e-g**) were found. Scale bar: 50 μ m

and rd1 (Figure 1a). Immunohistology revealed that HDACs representing all three major classes were present in wt and rd1 retina at post-natal day (P) 11. Both class I HDAC2 (Figure 1a, d) and class II HDAC5 (Figure 1b, e) were prominently expressed in nuclei of the outer nuclear layer (ONL), inner nuclear layer (INL), and ganglion cell layer. In contrast, class III HDAC Sirt2 (Figure 1c, f) was expressed predominantly in non-nuclear structures including photoreceptor segments, neuritic processes in the ONL, and different INL cells. No obvious differences in expression or localization between wt and rd1 at P11 were detected for any of the HDACs.

rd1 photoreceptor nuclei show hypoacetylation. Acetylation of lysine residues was studied in wt and rd1 retinae using acetylation-specific antibodies (Abs). In the ONL of wt mice at P11, an Ab detecting general acetylation of lysine residues showed homogeneous staining of the photoreceptor population (Figure 2a-c). In contrast, the P11 rd1 ONL presented staining 'gaps' that contained unlabeled photoreceptor nuclei (Figure 2d-f). Such lack of staining encompassed decreased histone acetylation, as confirmed by several Abs directed against specific acetylated histones (Supplementary Figure 1). No obvious signs for hypoacetylation for any of the Abs used were found in the inner retina of neither wt nor rd1. Western blot (WB) of P11 in vivo retinae with the acetyl-lysine Ab (Figure 2g) mainly revealed bands between 12 and 17 kDa that corresponded to the reported molecular weights of different histones and showed numerically decreased acetylation levels in the rd1 retina (wt. 100%; *rd1*: 89.8% \pm 40, n = 3).

HDAC activity is increased in rd1 photoreceptors. To investigate whether the decreased rd1 photoreceptor acetylation was related to increased activity of HDACs, we adapted a technique originally intended to measure tissue homogenates¹⁸ for the use on retinal tissue sections.

Whole fixed retinal cryosections revealed deacetylation activity. By specifically blocking either HDACs I/II with TSA or HDAC III with NAM, we were able to distinguish the relative contributions of different classes of HDACs to the total activity. and to follow their kinetics with different substrate concentrations. As proof of principle for the HDAC assay, we determined the K_m values for total HDAC activity on retinae of rd1 and wt mice (Supplementary Figure 2). The K_m value for the fluorogenic substrate of 83 μ M at 500 μ M NAD⁺ is in good agreement with previous reports.¹⁹ When studied with cellular resolution, both wt and rd1 tissue displayed HDAC activity in photoreceptor segments. All nuclear layers of wt retina were essentially devoid of visible activity (Figure 3a). In contrast, the rd1 ONL carried a subset of cells, displaying typical nuclear morphology of photoreceptors, with highly elevated HDAC activity (Figure 3b). Inhibition of either class I/II HDACs (Figure 3c) or HDAC III (Figure 3d) suggested that most of the excessive HDAC activity in rd1 photoreceptor originated from HDAC I/II. Together, these results implied that rd1 photoreceptor hypoacetylation was due to an excessive activation of HDACs, and most likely HDAC I/II.

HDAC activity and cell death. To test whether the high HDAC activity observed in *rd1* photoreceptors was related to



Figure 2 Decreased acetylation in *rd1* photoreceptors *in vivo*. At P11 immunofluorescence for acetylated lysine residues (green) on *wt* (**a**) and *rd1* (**d**) retinae revealed a number of DAPI-stained *rd1* photoreceptor nuclei (**b**, **e**; blue) with very low levels of protein acetylation. (Merged pictures in **c**, **f**, arrowheads indicate nuclei showing low acetylation levels.) WB with the same Ab mainly labeled bands corresponding to the molecular weight of histones (**g**). Scale bar: 20 μ m



Figure 3 HDAC activity in the retina *in vivo*. HDAC activity was detected *in situ* in particular in the segment layers of both *wt* (**a**) and *rd1* (**b**) retina. In contrast to *wt*, *rd1* retina displayed a subset of ONL cells with strongly increased HDAC activity. Activity of HDAC I/II (**c**) was studied by inhibition of HDAC III with NAM. Conversely, sirtuin activity (**d**) was assessed by inhibiting HDACs I/II with TSA. Combined inhibition with TSA and NAM was used as negative control (**e**). Scale bar: 50 μm

their degeneration, staining for acetylated lysine was combined with the TUNEL assay for dying cells. Most of the cells lacking acetylation (94.3% \pm 7.3 S.E.M., n=3) were also TUNEL positive (Figure 4a-c). In another co-labeling experiment, rd1 HDAC activity colocalized only partially $(32.5\% \pm 6.7 \text{ S.E.M.}, n=3)$ with TUNEL-positive cells (Figure 4d-f) implying that the activity generally preceded the final stages of cell death. To confirm this hypothesis, we assessed the number of HDAC activity positive cells in the ONL of wt and rd1 retinas at various time points during the second post-natal week (i.e., during onset and peak of rd1 degeneration). The analysis of the temporal progression of HDAC activity showed a marked increase in the number of HDAC activity positive cells at P9, with a peak in activity already at P11 (Figure 4g). Comparison with previously published data on cell death (TUNEL assay) and PARP activity⁹ indicated that the peak in HDAC activity preceded the peak of the degeneration by ~ 2 days. Overall, this suggested that an acetylation/deacetylation imbalance might have an important role in rd1 photoreceptor degeneration.

HDAC I/II inhibitors protect *rd1* **photoreceptors.** We then exposed retinal explants in a short-term culture paradigm (until P11) to inhibitors specific for the three main classes of HDACs and evaluated the outcome on photoreceptor viability using the TUNEL assay.

The HDAC I/II inhibitor TSA (1 μ M) did not affect the number of TUNEL-positive cells in the ONL of P11 wt retinal cultures (TSA: $1.6 \pm 0.2\%$, n=6 retinal cultures from six different animals *versus* vehicle controls: $1.7 \pm 0.2\%$, n = 9; Figure 5a, b). In the *rd1* retina, however, $1 \mu M$ TSA significantly decreased the rate of cell death (TSA: $2.2 \pm 0.4\%$, n = 12 versus vehicle: $3.5 \pm 0.2\%$, n = 27, P < 0.001; Figure 5c, d; quantification in panel e). A second HDAC I/II inhibitor, Scriptaid (6 μ M), also conferred a significant neuroprotective effect to rd1 photoreceptors (Scriptaid: $2.6 \pm 0.1\%$, n=6, versus vehicle: $3.5 \pm 0.2\%$, n = 27, P < 0.05). The HDAC III inhibitor NAM (200 μ M and 1 mM) had no measurable effect on photoreceptor viability (NAM 200 μ M: 3.9 ± 0.2%, n=5*versus* vehicle: $3.5 \pm 0.2\%$, n = 27; Figure 5e). Neither of the above treatments affected the number of photoreceptor rows (quantification in Figure 5e). Successful in vitro HDAC



Figure 4 Protein acetylation and temporal progression of HDAC activity and cell death *in vivo*. At P11, immunostaining for acetylated lysine residues (**a**, green) in *rd1* ONL showed characteristic "gaps", which were partly filled by TUNEL staining (red) for dying cells (**b**) (merged in **c**), suggesting hypoacetylation to contribute to photoreceptor cell death. Conversely, the HDAC activity assay (**d**, **f**) colocalized in part with TUNEL staining at P11 (**e**) in the *rd1* ONL (merged in **f**). Scale bar: 20μ M. The graph in **g** shows the temporal progression of HDAC activity (blue line) in *wt* and *rd1* ONL during the second postnatal week, and relates it to the time-course for TUNEL-positive cells (red). Note that the peak of HDAC activity markedly precedes the peak of TUNEL. $n \ge 3$ for each time point and genotype, data for TUNEL assay were previously published in Paquet-Durand *et al.*, 2007 and are shown here for reference purposes only

inhibition by TSA was confirmed using both a tissue-based analysis (WB for acetylated lysine; Figure 5f) and an *in situ* approach (HDAC activity; Supplementary Figure 3).

Although we did not observe any negative properties of TSA in our cultures, a previous study on acute *wt* retinal explants showed proapoptotic effects of TSA at early developmental stages.¹⁶ To address the possibility of a transient TSA effect on retinal cell viability, we treated P5 *rd1* retinal explants with 1 μ M TSA for 24 h, but this did not affect the percentage of TUNEL-positive cells in the P6 *in vitro* retina (TSA: 1.7 ± 0.2%, vehicle: 1.5 ± 0.3%; *n*=3; Supplementary Figure 4). Furthermore, when extending the culture period for another 5 days without TSA (P5+1+5=P11), to match with the end point used in our previous experiments, the treated *rd1* retina again showed a markedly reduced number of TUNEL-positive cells (TSA: 1.0 ± 0.2%; vehicle: 3.8 ± 0.5%, *n*=3, *P*<0.05). At the same time, the number of photoreceptor rows was not significantly affected by the treatment (TSA: 7.1 \pm 0.2, vehicle: 7.5 \pm 0.1; n=3).

To test whether the strong neuroprotective effect of TSA in the different short-term paradigms would translate into improved long-term survival of photoreceptors, *in vitro* treatment was prolonged until P28. TSA treatment almost doubled the number of surviving *rd1* photoreceptor rows (TSA: 4.1 ± 0.3 ; vehicle: 2.1 ± 0.2 , n = 6, P < 0.001; Figure 5i).

Expression of rhodopsin was then analyzed as an indication of rod photoreceptor identity. In *wt* retina, *in vivo* rhodopsin expression is restricted to the outer segments of rod photoreceptors, whereas in rd1 retina, where outer segments do not develop properly, it is partly mislocalized and also observed throughout the cytoplasm of photoreceptors.²⁰ This phenomenon intensifies under *in vitro* conditions where



Figure 5 HDAC inhibition reduces photoreceptor cell death *in vitro*. Short-term retinal explant cultures from *rd1* and *wt* animals were exposed to control conditions or 1 μ M TSA from P7 to P11 (**a-d**). In untreated specimens (**a**, **c**), TUNEL assay (green) showed the characteristic increase of dying cells in *rd1* ONL compared with *wt*. TSA treatment (**b**, **d**) reduced the number of degenerating photoreceptors in *rd1*, but did not alter viability of *wt*. The bar graph (**e**) summarizes effects of TSA, 6 μ M Scriptaid, and 200 μ M NAM on photoreceptor viability. HDAC I/II inhibitors, TSA and Scriptaid, significantly reduced the amount of dying cells, whereas HDAC III inhibitor NAM had no effect on cell survival. Immunoblotting for acetylated lysine residues (**f**) showed an increase in protein acetylation after TSA treatment in both *wt* and *rd1* retinae. Long-term cultures of *rd1* retinae (until P28; **g**, **h**) treated with TSA showed a marked increase in the number of rhodopsin-positive cell bodies and overall size of their ONL when compared with untreated situation (quantification in **i**). DAPI (blue) was used as nuclear counterstain; actin immunoblotting was used as loading control in **f**; $n \ge 6$, Scale bars in **d** and **h** are 50 and 20 μ M respectively

rhodopsin mislocalization is observed in both *wt* and *rd1* photoreceptors (Supplementary Figure 5). In long-term TSA-treated explants, rhodopsin expression was found in most of the surviving *rd1* photoreceptors, whereas in the untreated situation most remaining ONL cells were rhodopsin negative and most likely predominantly cones (Figure 5g, h). Significantly, rhodop-sin-positive photoreceptor outer segments were observed in TSA-treated specimens, when at the same time in the untreated situation, outer segments were completely absent. This supported the idea that TSA indeed upheld survival of rod photoreceptors and promoted growth of *rd1* outer segments.

All in all, TSA treatment in both short- and long-term retinal culture demonstrated a strong prosurvival effect, whereas NAM had no such protective effects. The experiments therefore suggested a major contribution of HDAC I/II, but not III, to mutation-induced *rd1* photoreceptor death.

HDAC I/II regulate PARP activity. As we had previously found PARP activity to be involved in rd1 photoreceptor cell

death,⁹ we wanted to investigate a possible interaction between HDACs and PARP. When immunostainings for acetylated lysines and for poly-ADP-ribose (PAR) – the product of PARP activity – were combined, the merged images revealed PAR-positive photoreceptor nuclei exclusively in cases where acetylation was absent (Figure 6a–d). Together with the data on the progression of HDAC activity (Figure 4g), this suggested that hypoacetylation and hence HDAC activity was preceding PARP activity.

We then tested whether HDAC I/II inhibition by TSA indirectly also affected PARP activity. With respect to PAR immunostaining, there was a moderate amount of positive photoreceptor nuclei in *wt* retinal explant cultures at P11 (*wt* vehicle: $0.25\% \pm 0.1$, n=3), and a comparatively much higher number in *rd1* explants (*rd1* vehicle: $0.9\% \pm 0.1$; Figure 6e, g). The presence of PAR-positive photoreceptors in *wt* explants is likely due to the stress that these undergo during culture. In both *wt* and *rd1* explants, the number of PAR-positive cells was reduced by TSA treatment, and this reduction was



Figure 6 HDAC and PARP activities are connected. In *rd1* P11 *in vivo* specimens, a subset of ONL nuclei showed unusual chromatin condensation as evidenced by DAPI nuclear counterstain (**a**, blue). In most of these cells a strong accumulation of poly-ADP-ribose (PAR) (**b**, red) colocalized with hypoacetylation (**c**, green; merged in **d**; white arrowheads), suggesting a functional connection between acetylation and PARP activity. In short-term retinal explant cultures at P11, photoreceptors showing PAR accumulation were observed in untreated *wt* (**e**) and *rd1* retina (**g**), their number being much higher in the latter case. This number was strongly reduced by TSA treatment in both *wt* (**f**) and *rd1* retina (**h**; quantification in **i**), implying that activation of PARP occurred downstream of HDAC I/II activity. n = 3; Scale bar in **d**, $\mathbf{h} = 20 \, \mu$ M. Some of the PAR immunostaining between ONL and INL relates to IGG staining in blood vessels

statistically significant in the *rd1* situation (*rd1* TSA: $0.3\% \pm 0.2$, n=3, P<0.05; Figure 6f, h). These results supported the notion that activation of PARP in *rd1* photoreceptors occurred downstream of HDAC I/II activity.

Discussion

The activity of HDACs has previously been connected to a variety of different cellular processes, notably regulation of gene expression, cytoskeletal rearrangements, division, and differentiation.¹³ Here, we show that HDAC activity was causally related to inherited photoreceptor cell death in the *rd1* mouse and that this detrimental effect was tied to HDACs I/II. HDACs III did not seem to be involved in the degeneration, which corresponds to observations on weakened HDAC III action in degenerating retina.²¹ The strong neuroprotection afforded by HDAC I/II inhibitors in both short- and long-term retinal explant cultures proposes them as novel lead compounds to prevent or delay retinal neurodegeneration.

HDAC and gene regulation. Given the extensive effects of acetylation/deacetylation on gene regulation, the observed HDAC hyperactivation would likely result in significantly altered

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rd1 gene transcription, which matches with previous microarray data.⁵⁻⁷ On the other hand, we can at present not exclude the possibility that the observed histone deacetvlation was due in part also to a decreased activity of HATs. Our observations that rd1 gene alterations include downregulation of the transcription factor CREB^{6,8,22} is of particular interest here, as CREB not only appears to confer neuroprotection^{6,8,22} but also has several links to cellular acetylation events. For example, different HDAC I/II isoforms can recruit protein phosphatase-1 to dephosphorylate and inactivate CREB.²³ which might be related to its downregulation in the rd1 retina. Moreover, CREB target gene transcription is facilitated by its co-factor CREB-binding protein (CBP), which has HAT activity,24 and therefore connects CREB activation with histone acetylation. Accordingly, some of the effects on neuronal gene regulation seen when acetylation is increased by HDAC inhibition may be routed through enhanced activity of CREB and CBP.²⁵ The expression of opsin and other photoreceptor genes is controlled by the photoreceptorspecific transcription factor cone-rod-homeobox (CRX).²⁶ In conjunction with histone acetylation and CBP, CRX drives expression of these critical photoreceptor genes in Y79 retinoblastoma cells and in the mouse retina.15,27

Cross-talk between HDAC and PARP activity. PARP activity is involved in DNA damage repair and hence often seen as a benign factor, although excessive PARP activity may compromise cellular viability.¹⁰ Increased PARP activity, as seen in rd1 photoreceptors,⁹ consumes NAD⁺ and generates NAM, which in turn negatively regulates HDAC III activity and thereby favors acetylation of its target proteins.¹⁴ As HDAC IIIdependent deacetylation of the automodification domain of PARP blocks its activity,²⁸ low NAD⁺ levels could trigger a positive feedback loop accounting for both the high PARP activity and the low HDAC III activity observed in rd1 photoreceptors. Yet other reports demonstrate that acetylation by p300/CBP promotes the ability of PARP-1 to act as a co-activator in transcription³⁰, whereas acetylation by histone acetyltransferases PCAF and GCN5L reduces PARP-2 catalytic activity.^{29,30} The findings highlight a possibility for hyperactive HDAC I/II to intervene with various PARP types, perhaps causing abnormal stimulation of these enzymes. In such a scheme, HDAC I/II activity would be upstream of PARP activation in rd1 photoreceptor degeneration, which is in accord with our results, where TSA treatment reduced accumulation of PAR. In addition, PARP activity accompanies the event of dying, TUNEL-positive photoreceptors and colocalizes to a large extent with them,9 whereas in this study, TUNEL colocalization with HDAC activity was only minor. This again implies that HDAC activity was upstream of PARP activation.

HDAC activity in cell death and survival. Experimental evidence suggests that both overactivation of HDACs I/II or their inhibition promotes cell death.¹³ Similarly, HDAC III activity can have both pro- and antiapoptotic effects depending on cell type and proliferation state,³¹ and the right balance between the activities of different HDAC classes therefore seems to be crucial for maintaining cellular viability.¹³ In addition, individual members of each HDAC class could have distinct cell typespecific roles,³² perhaps related to their expression levels, which may in part explain the opposing effects of HDAC inhibitors observed in different neurodegeneration paradigms.¹² The protective effects of TSA and Scriptaid comply with a number of studies on post-mitotic neurons, where HDAC inhibitors also conferred neuroprotection, 12,33,34 possibly in part due to the prevention of oxidative stress³⁵ and reactivation of CREB signaling.^{36,37} Furthermore, it is conceivable that in the rd1 situation HDAC inhibition may induce expression or increase the stability of neuroprotective genes and proteins that are not directly related to the pathological mechanism. In this context, the chaperone HSP90, which is a target for HDACdependent deacetylation, appears to have a prominent role in neurodegeneration. Acetylation at its K294 residue inhibits HSP90³⁸ and hence HDAC inhibitors indirectly also block HSP90 activity.³⁹ Interestingly, HSP90 inhibition induces an upregulation of the neuroprotective chaperone HSP70 and strongly increases neuronal survival.40,41 Our finding that decreased histone acetylation combines with increased HDAC activity corresponds to very similar observations made in motorneurons in a mouse model for amyotrophic lateral sclerosis, where HDAC inhibition also resulted in neuroprotection.36 In the in vivo retina, HDAC inhibition has been shown to promote survival of ganglion cells after optic nerve crush.37

Conclusion

The mechanisms governing inherited photoreceptor cell death have remained elusive to date.1 Our discovery that HDAC activity was involved in photoreceptor degeneration may thus help to close a major gap of knowledge between the immediate effects of a single gene mutation² and the observed massive changes at the transcriptional level.⁶ Moreover, increased HDAC activity appeared to be responsible for an activation of PARP in degenerating rd1 photoreceptors. The exact interplay between PARP and HDAC activity warrants further studies, as it may potentially explain crucial events in the pathological process. This study not only brings up HDAC activity as a 'missing link,' uniting several other findings on the mechanisms of photoreceptor cell death, but also highlights HDACs as novel targets for the development of neuroprotective therapies aimed to halt or delay inherited retinal degeneration in humans.

Material and Methods

Animals. Animals were housed under standard white cyclic lighting, had free access to food and water, and were used irrespective of gender. C3H *rd1/rd1* (*rd1*) and control C3H *wt* mice were used.⁴² All procedures were performed in accordance with the local ethics committee in Lund (permits M225-04 and M242-07), the Tübingen University (§4 registration from 23–01–08), and the ARVO statement for the use of animals in ophthalmic and visual research. All efforts were made to minimize the number of animals used and their suffering. Because of the critical changes at P11,^{22,43} most comparisons between *rd1* and *wt* were carried out at this age. Day of birth was considered as P0.

Microarray analyis. Details of the transcription assay performed on a custommade mouse gene chip were described in Azadi *et al.*⁶ Two times up- or downregulation in at least three out of five hybridizations was considered as significant variation. Results not pertaining to this study are published elsewhere.

In vitro retinal explant cultures. Retinae from P5 *rd1* and *wt* animals were used to generate retinal explants as described before.^{4,44} In brief, animals were killed and the eyes enucleated in an aseptic environment. Afterwards, the entire eyes were incubated in R16 serum-free culture medium (Invitrogen Life Technologies, Paisley, UK; 07490743A) containing 0.12% proteinase K (MP Biomedicals, Solon, OH, USA; 193504) at 37 °C for 15 min, to allow preparation of retinal cultures with RPE attached. Proteinase K was inactivated with 10% fetal calf serum (Invitrogen Life Technologies; PET10108165) in R16 medium, and the eyes dissected aseptically in a Petri dish containing R16 medium. The anterior segment, lens, vitreous, sclera, and choroids were carefully removed, and the retina was cut perpendicular to its edges, resulting in a cloverleaf-like shape. Subsequently, the retina was transferred to a Millicell culture dish filter insert (Millipore AB, Solna, Sweden; PIHA03050) with the retinal pigment epithelium layer facing the membrane. The insert was put into a six-well culture plate and incubated in R16 nutrient medium at 37 °C. Every second day, the full volume of nutrient medium, 1.5 ml per dish, was replaced with fresh medium.

The first 2 days, the retina was left in R16 culture medium without treatment to adapt to culture conditions. At P7, cultures were either exposed to different treatments (TSA 1 μ M, Scriptaid 6 μ M, NAM 200 μ M, and 1000 μ M; all from Sigma, Munich, Germany), or kept as untreated control. NAM is a constituent of the culturing medium and present at about 22 μ M, which should be less than half the reported IC₅₀ value in cell-free studies.⁴⁵ Explants were cultured until P11 for short-term cultures (P5 + 2 + 4), or until P28, for long-term cultures (P5 + 2 + 2). Culturing was stopped by 2 h fixation in 4% paraformaldehyde (PFA), washed 4 × 15 min in phosphate-buffered saline (PBS), then cryoprotected first with PBS + 10% sucrose, and subsequently with PBS + 25% sucrose. Explants were frozen and embedded in Jung tissue freezing medium (Leica Microsystems, Nussloch, Germany) for cryosectioning (12 μ m).

HDAC assay. HDAC activity assays were performed on cryosections of 4% PFA P11 fixed eyes. The assay is based on an adaptation of the Fluor de Lys Fluorescent Assay System (Biornol, Hamburg, Germany). Retinal sections were exposed to

different concentrations of Fluor de Lys-SIRT2 deacetylase substrate (Biomol) with 2 or 100 μ M TSA (Sigma, Steinheim, Germany), 2 mM NAM (Sigma), and 500 μ M NAD ⁺ (Biomol) in assay buffer (50 mM Tris/HCl, pH 8.0; 137 mM NaCl; 2.7 mM KCl; 1 mM MgCl₂; 1 mg/ml BSA) and 0.1% NP40 and 0.5 × Developer II (Biomol). Images were captured at indicated time points with a fixed exposure time of 2 s using a Zeiss (Jena, Germany) Axiophot microscope (× 20 magnification) and Zeiss Axiovision 4.2 software; densitometry was performed with the aid of ImageJ (National Institutes of Health, Bethesda, MD, USA). The reaction rate was determined in at least four independent experiments, as the slope of the linear regression fitted to the data points in the linear range of a time course, using SigmaPlot (Systat software, Erkrath, Germany) software. For the calculation of K_m values, the slope of time courses with different substrate concentrations was plotted against the corresponding substrate concentrations; a hyperbolic regression fitted to the data points revealed K_m values and V_{max} .

In situ deacetylation activity was determined on retinal sections incubated for 3 h with 200 μ M Fluor de Lys-SIRT2 deacetylase substrate in assay buffer with/without inhibitors. Sections were then washed in PBS and fixed in methanol at -80 °C for 20 min. \times 0.5 developer (Biomol) in assay buffer was applied and pictures taken immediately in an Apotome microscope (Zeiss), at \times 20 using the DAPI filter.

TUNEL assay. The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed on cryosections from treated and untreated *rd1* retinas, using an *in situ* cell death detection kit conjugated with tetramethyl-rhodamine or fluorescein isothiocyanate (Roche Diagnostics, Mannheim, Germany). For controls, terminal deoxynucleotidyl transferase enzyme was either omitted from the labeling solution (negative control), or sections were pretreated for 30 min with DNAse I (Roche Diagnostics, 3 U/mI) in 50 mM Tris-HCI, pH 7.5, 1 mg/mI BSA to induce DNA-strand breaks (positive control). Negative control showed no staining at all, whereas positive control stained all nuclei in all layers of the retina.⁹

Immunostaining. Frozen retinal sections from P11 animals or cultured retinae were dried for 30–60 min at 37 °C. Subsequently, the tissue was rehydrated in PBS, and preincubated for 1 h at room temperature (RT) in blocking solution, containing 10% normal serum, and 0.1 or 0.3% Triton in PBS (PBST). Immunohistochemistry was performed overnight at 4 °C, using primary Abs (acetylated lysine; acetylated H2A, H2B, H3, H4; Sirt2; HDAC2; HDAC5; PAR) diluted 1:100 in blocking solution. Primary Abs were purchased from Cell Signaling (Danvers, MA, USA) except PAR Ab (Alexis Biochemicals, Lörrach, Germany) and Rhodopsin Ab (Millipore, Schwalbach, Germany). The tissue was rinsed with PBST, and incubated for 1 h with a corresponding secondary Ab, Alexa 488 (1:200–1:750, Invitrogen), diluted in PBST. Sections were rinsed in PBS, and mounted in Vectashield with DAPI (Vector Laboratories, Burlingame, CA, USA).

Western Blot. Retinae from P11 animals were enucleated and stored at -80 °C. Later on, the tissue was homogenized in buffer (10 mM Tris, 1 mM EDTA, 150 mM NaCl. 1 mM Na₃VO₄, 50 nM okadaic acid, 2% SDS, 10% glycerol, 0.0625 M Tris-HCl, and protease inhibitor cocktail 10 μ l/ml (Calbiochem, Darmstadt, Germany); pH 6.8) with a Heidolph DIAX 600 homogenizer (Heidolph, Schwabach, Germany) or a manual homogenizer (glass to glass). Retinal explant cultures, both treated and untreated with TSA, from wt and rd1 mice were homogenized using the same process. Bradford assay was used to measure protein concentration. Proteins were then separated by SDS-PAGE 4-12% gradient gel (at 55 V), and transferred to a PVDF membrane (Amersham Biosciences, Buckinghamshire, UK). Membranes were incubated in Roti block (Roth, Karlsruhe, Germany) blocking buffer for 3 h at RT. After washing, primary Abs (acetylated lysine; acetylated H2A, H2B, H3, H4; actin) were added at a dilution of 1:1000 in buffer containing TBS-T and 5% dried milk (Roth), and incubated overnight at 4 °C. Primary Abs were purchased from Cell Signaling, except for actin Ab which was from Chemicon (Schwalbach, Germany). After washing, the membranes were treated with buffer containing HRP-conjugated secondary Ab (Amersham Biosciences) overnight at 4 °C. The membranes were developed using Hyperfilm (Amersham Biosciences) detection system. Quantification was performed after film scanning using ImageJ (NIH).

Microscopy, cell counting, and statistics. Morphological observations and routine light microscopy were performed on a Zeiss Imager Z1 Apotome Microscope, equipped with a Zeiss Axiocam digital camera. Images were captured using Zeiss Axiovision 4.7 software; image overlays and contrast enhancement were performed using Adobe Photoshop CS3. Images shown in figures are representative for least three different animals for each genotype/treatment. Percentages of TUNEL-positive cells were assessed and calculated in a blinded manner as reported previously.^{4,9} The mean value for photoreceptor rows in the ONL after *in vitro* culture was determined using DAPI nuclear counterstaining. Values are given as mean \pm S.E.M. Statistical significance was tested using one-way ANOVA with Bonferroni correction and Prism (GraphPad Software, San Diego, CA, USA), significance levels were P < 0.05 (*), P < 0.01 (**), and P < 0.001 (***).

Conflict of interest

The authors declare no conflict of interest.

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