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Accessibility
Selective Sirt2 inhibition by ligand-induced rearrangement of the active site

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Sirtuins are a highly conserved class of NAD\(^+\)-dependent lysine deacylases. The human isotype Sirt2 has been implicated in the pathogenesis of cancer, inflammation and neurodegeneration, which makes the modulation of Sirt2 activity a promising strategy for pharmaceutical intervention. A rational basis for the development of optimized Sirt2 inhibitors is lacking so far. Here we present high-resolution structures of human Sirt2 in complex with highly selective drug-like inhibitors that show a unique inhibitory mechanism. Potency and the unprecedented Sirt2 selectivity are based on a ligand-induced structural rearrangement of the active site unveiling a yet-unexploited binding pocket. Application of the most potent Sirtuin-rearranging ligand, termed SirReal2, leads to tubulin hyperacetylation in HeLa cells and induces destabilization of the checkpoint protein BubR1, consistent with Sirt2 inhibition in vivo. Our structural insights into this unique mechanism of selective sirtuin inhibition provide the basis for further inhibitor development and selective tools for sirtuin biology.
**Identification and crystallization of SirReal inhibitors.** In search for new sirtuin inhibitors, we screened an internal compound library using an *in vitro* assay based on a fluorophore-labelled acetyl-lysine derivative for human Sirt1–3. In this screening campaign, a family of aminothiazoles that we have termed Sirtuin-rearranging ligands (SirReals) was discovered as potent, Sirt2-selective inhibitors. Of these, SirReal2 (1) showed the most promising inhibitory properties (Fig. 1a,b). AGK2 was used as a reference inhibitor. Under the same assay conditions it is 38-fold less potent with an IC50 of 15.4 ± 0.7 μM. The activity of Sirt1 or Sirt3 was not affected at 50 μM. Additional confirmation of Sirt2-selective *in vitro* inhibition and binding by SirReal2 was obtained by using non-labelled peptidic substrates in a high-performance liquid chromatography (HPLC)-based conversion assay (Fig. 1c, Supplementary Fig. 1b) and from thermal stability assays, where the presence of SirReal2 led to increased melting temperatures due to ligand-induced stabilization of the protein (Fig. 1d). SirReal2 only inhibits Sirt2 potently with an IC50 value of 0.4 μM. The crystal structures of SirReal2 in complex with Sirt2 (PDB codes 5DKD and 5DKI) revealed a potent, Sirt2-selective small-molecule inhibitor with drug-like properties. The basis for the high potency and unprecedented isotype selectivity is a ligand-induced structural rearrangement of the active site, exploiting an adjacent binding pocket. Along with kinetic studies, the structures give insight into a unique and isotype-selective inhibition mechanism. The relevance of the observed biochemical activity is further supported by cellular studies.

**Results**

**Figure 1 | SirReal2 selectively inhibits Sirt2 in a dose-dependent manner.** (a) Chemical structure of SirReal2 (1). (b) Representative dose–response curve for Sirt1–3 and SirReal2 using the substrates ZMAL (2-Lys(Acetyl)-AMC, Sirt1–2) resp. Fluor-de-Lys (Sirt3). Compared with the peptide-HPLC assay, SirReal2 was slightly less potent using ZMAL with an IC50 value of 0.4 μM. Data are presented as mean ± s.d. (n = 3). (c) *In vitro* inhibition data for SirReal2 (Sirt1–3: 100 μM; Sirt4–6: 200 μM) in an assay using non-labelled acetyl-lysine oligopeptide as a substrate (Sirt1–4, acetyl-lysine substrate; Sirt5, succinyl-lysine substrate; Sirt6, myristoyl-lysine substrate). A solution containing DMSO was used as a negative control, a solution with nicotinamide (NCA, 200 μM or 1 mM) was used as a positive control. Only the activity of Sirt2 is substantially reduced in the presence of SirReal2. Data are presented as mean ± s.d. (n = 2) (d) Representative thermal stability plots for Sirt2 in the presence of SirReal2 (25 μM) and either the cofactor NAD+ (5 mM) or an acetyl-lysine H3 peptide (5 mM). The presence of NAD+ or of an acetyl-lysine peptide substrate enhances the stability of the Sirt2–SirReal2 complex (n = 3). Representative thermal stability plots of Sir2 in the absence of SirReal2 and in the presence of NAD+ or an acetyl-lysine oligopeptide are shown in Supplementary Fig. 1d. Rel., relative.
of 140 nM and has very little effect on the activities of Sirt3-5. Only the activity of Sirt1 (22% inhibition at 100 µM) and Sirt6 (19% inhibition at 200 µM) are slightly affected at higher SirReal2 concentrations, making SirReal2 one of the most selective sirtuin inhibitors up to date. However, any attempts to identify a putative-binding site and to rationalize initial structure–activity relationships by docking to available X-ray structures of Sirt2 were not successful. We, therefore, proceeded to determine the structures of Sirt2-inhibitor complexes by protein X-ray crystallography.

For that, we used a truncated form of Sirt2_{56–356} lacking the flexible N- and C termini. To validate the suitability of our expression construct, we also crystallized this truncated form of Sirt2 in the presence of ADP ribose (ADPR) and the physiological inhibitor nicotinamide (NCA, Supplementary Fig. 2, structure termed Sirt2–ADPR–NCA). As the binding mode of these ligands corresponded to other published sirtuin structures in complex with NCA^{30}, we concluded that our expression construct was suitable for the investigation of Sirt2–ligand interactions. Further thermal stability experiments indicated an additional stabilization of the Sirt2–SirReal2 complex in the presence of either NAD^{+} or a peptidic acetyl-lysine substrate (Fig. 1d). These findings were the key to a successful crystallization of Sirt2 in complex with SirReal2 that was only achieved in the presence of either substrate or cosubstrate.

**Overall structure of Sirt2–SirReal2 complexes.** We solved the structure of Sirt2 in complex with SirReal2 and the cosubstrate NAD^{+} (structure termed Sirt2–SirReal2–NAD^{+}) as well as in complex with SirReal2 and an acetyl-lysine peptide derived from histone H3 (residues 11–17, structure termed SirReal2–SirReal2–H3). Both Sirt2–SirReal2 crystals belonged to different monoclinic space groups and contained one monomer per asymmetric unit. They had the two-domain structure typical for sirtuins—a larger domain with a Rossmann fold and a smaller zinc-binding domain that are separated through a large groove that constitutes the active site (Fig. 2a). The structures are highly similar (root mean squared deviation, r.m.s.d. (C_\text{α} atoms) = 0.8 Å) with the main differences in the cofactor-binding loop and its adjacent residues (r.m.s.d. (C_\text{α} residues 92–115) = 1.3 Å). In addition, we observed the Sirt2-specific insertion (residues 289–304) that mediates crystal contacts as was reported for the Sirt2–ADPR complex (ADPR, PDB-ID 3ZGV^{19}) and apo-Sirt2 (PDB-ID 1BJF, refined

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**Figure 2** | **SirReal2 functions as a molecular wedge locking Sirt2 in an open conformation.** (a) Overlay of Sirt2–SirReal2–NAD^{+} (slate blue) and Sirt2–SirReal2–H3 (light grey). Both structures are very similar (r.m.s.d. (C_\text{α} atoms) = 0.8 Å) and feature an open conformation. The active site is indicated by small grey dots. (b) Superposition of Sirt2–SirReal2–NAD^{+} (slate blue) with Sirt2-apo (PDB-ID 3ZGO, salmon, residues 34–45 are omitted for better clarity). Both structures feature an open state despite major structural differences in the zinc-binding domain. (c) Superposition of Sirt2–SirReal2–NAD^{+} (slate blue) with the Sirt2–ADPR complex (PDB-ID 3ZGV, yellow, residues Tyr139–Gly141 of one hinge loop were not defined in the electron density map). The structures display major conformational differences in the orientation of the zinc-binding domain. While the ADPR complex is in a closed state, Sirt2–SirReal2–NAD^{+} adopts an open state. (d) Superposition of Sirt2–SirReal2–NAD^{+} with Sirt2 in complex with a macrocyclic peptide inhibitor S2iLS (PDB-ID 4L3O, green). Similar to the Sirt2–ADPR complex, the Sirt2–S2iLS complex assumes a closed conformation. While the Rossmann fold domain is very similar in both structures, major structural differences can be seen at the zinc-binding domain and at the Sirt2-specific insertion. (e) Close-up view on the active site using the superposition shown in a. SirReal2 (Sirt2–SirReal2–NAD^{+}, light pink sticks; Sirt2–SirReal2–H3, light cyan sticks) occupies the extended C-site of Sirt2. Binding of SirReal2 neither prevents binding of the acetyl-lysine substrate (light blue sticks) nor the cosubstrate NAD^{+} (light orange sticks). The cofactor-binding loop of both structures is omitted for clarity.
3ZGO, 18,19). The cofactor-binding loop in both Sirt2–SirReal2 complexes adopts a conformation similar to the one observed in Sirt2 in complex with the product analogue ADPR (PDB-ID 3ZGV).

When compared with the available Sirt2 structures (apo-Sirt2: PDB-ID 1B8F, 3ZGO; Sirt2-ADPR: PDB-ID 3ZGV; Sirt2–S2iL5 peptide: PDB-ID 4L3O, 28) the zinc-binding domains of the Sirt2–SirReal2 structures adopt a conformation similar to the one in apo-Sirt2 (Fig. 2b–d, Supplementary Fig. 3a–c). On binding of the acetyl-lysine peptide substrate, the zinc-binding domain rotates towards the Rossmann fold domain. This has been termed as the ‘closure’ of the two domains and can be observed in several other human and bacterial sirtuin structures in complex with an acetyl-lysine peptide substrate,20,21,31. This domain closure induces the formation of the acetyl-lysine-binding tunnel and the β-staple motif that mediates the acetyl-lysine peptide substrate-sirtuin interactions.32 Despite the absence of an acetyl-lysine peptide substrate, the Sirt2–ADPR complexes also adopt the closed conformation. This is due to the Sirt2-specific insertion that acts as a pseudo-substrate in the crystal and binds to the acetyl-lysine-binding site of a neighbouring Sirt2 molecule.

Despite the ‘open’ conformation of the Sirt2–SirReal2 structures, SirReal2-inhibited Sirt2 adopts a substantially different structure from the one observed in Sirt2–apo (r.m.s.d. (Cα atoms) = 1.4 Å, Fig. 2b), the complex of Sirt2 and ADPR (r.m.s.d. (Cα atoms) = 1.6 Å, Fig. 2c) and the complex of Sirt2 and the S2iL5 peptide (r.m.s.d. (Cα atoms) = 1.7 Å, Fig. 2d). Our structures feature a completely new and unexpected SirReal2 conformation, where SirReal2 functions as a ‘molecular wedge’ that traps Sirt2 in the open conformation even in the presence of an acetyl-lysine peptide substrate. We call this a ‘locked open’ state.

**SirReal2 occupies a yet-unexpected binding pocket.** SirReal2 binds to the active site of Sirt2 (Figs 2e and 3) at the interface of the Rossmann fold domain and the zinc-binding domain, the site of the deacylation of ε-amino groups of lysines. The active site of Sirt2 has previously been divided into different sites (Fig. 3a). The A- and B-pocket, respectively, bind the ADPR moiety, whereas the C-pocket binds the NCA of NAD+; NAD+ is able to adopt different conformations. However, only a kinked conformation where the NCA moiety of NAD+ occupies the C-pocket is considered productive for catalytic deacylation. The hydrophobic
acetyl-lysine-binding tunnel is formed by several phenylalanines and connects the NAD$^+$-binding site to the acetyl-lysine-binding site. The pocket adjacent to the C-pocket has been termed extended C-site (EC-site)\textsuperscript{33}.

The aminothiazole SirReal2 occupies this EC-site adjacent to the C-pocket, which is the physiological site for product inhibition by the feedback inhibitor NCA\textsuperscript{30} (Supplementary Fig. 2). It binds at this highly hydrophobic site in vicinity to the zinc-binding domain, where it does not interfere with the binding of NCA or the NCA moiety of NAD$^+$ (Fig. 3b–d). The naphthyl moiety of SirReal2 protrudes into the substrate channel and the dimethylmercaptopyrimidine substituent (DMP) induces the formation of a binding pocket beyond the EC-site. This region is formed by two loops (residues 136–144, residues 188–191) of the hinge region that connect the Rossmann fold domain with the zinc-binding domain. We refer to this binding pocket in the following as the ‘selectivity pocket’. The position of SirReal2 in the EC-site of Sirt2 is very similar in both structures with either NAD$^+$ or an acetyl-lysine peptide (r.m.s.d. of 0.47 Å), and we will primarily describe the binding of SirReal2 in the presence of NAD$^+$, as this structure likely represents the SirReal2-inhibited form of the enzyme. Structural comparison of the available Sirt2 structures with the Sirt2–SirReal2–H3 complex can be found in Supplementary Fig. 3.

Binding of SirReal2 to the EC-site is mainly driven by hydrophobic interactions (Fig. 3c,d). The naphthyl moiety of SirReal2 that protrudes into the acetyl-lysine-binding site is in van-der-Waals contacts with the NCA moiety of NAD$^+$, Phe131, Ile169, Ile232, Val233 and Phe234. In the selectivity pocket, the DMP moiety forms π–π-stacking interactions with Tyr139 and Phe190 in the selectivity pocket that is shaped by Ile93, Ala135, Leu138, Pro140, Phe143, Leu206 and Ile213. In addition, Pro94 hydrogen bonds via a structural water molecule (W40) to the carbonyl-O of SirReal2. Besides its interactions with the Sirt2 protein, the SirReal2 inhibitor also forms an internal hydrogen bond between the amide N–H and one of the pyrimidine nitrogens. This results in a rigid conformation with ideal complementarity to the active site of Sirt2.

**Figure 4 | SirReal1 selectively inhibits Sirt2 and functions as a molecular wedge to lock Sirt2 in an open conformation.**

(a) Chemical structure of SirReal1 (2). (b) Representative thermal stability plots for Sirt2 in the presence of SirReal1 (50 μM) and either the cofactor NAD$^+$ (5 mM) or an acetyl-lysine H3 peptide (5 mM). The presence of the cosubstrates enhances the stabilization of the SirReal2–Sirt2 complex (n = 3). Representative thermal stability plots of Sirt2 in the absence of SirReal1 are shown in Supplementary Fig. 1d. (c) Overlay of Sirt2–SirReal1–OTC (brown) with Sirt2 structures in complex with SirReal2 (Sirt2–SirReal2–H3, light grey; Sirt2–SirReal2–NAD$^+$, slate blue). All SirReal2–Sirt2 complexes share a high similarity (r.m.s.d. (C$\alpha$ atoms) = 0.44 Å to Sirt2–SirReal2–H3, 0.59 Å to Sirt2–SirReal2–NAD$^+$) and represent the locked open conformation. The active site is represented as grey dots. (d,e) SirReal1 (light yellow sticks) occupies the extended C-site in a very similar fashion as observed for SirReal2 (light blue in Sirt2–SirReal2–H3, light pink in Sirt2–SirReal2–NAD$^+$). Differences can be observed for the position of the side chains of Phe119, Phe235 and the acetyl-lysine peptides. The acetyl-lysine-binding site as well as the selectivity pocket are also the sites of major conformational changes compared with Sirt2–apo (PDB-ID 3ZGO) and Sirt2–ADPR (PDB-ID 3ZGV, see Fig. 3e). Hydrogen bonds are shown in dashed yellow lines. The cofactor-binding loop of d is omitted for clarity. A stereo image of the σ-weighted 2F$\sigma$ – F$\sigma$ electron density maps for SirReal1 and the Ac-Lys–OTC oligopeptide as well as σ-weighted F$\sigma$ – F$\sigma$ electron density OMIT maps of both ligands are shown in Supplementary Fig. 5b,d.

**Binding of SirReal2 to Sirt2 rearranges the active site.** The presence of SirReal2 results in a rearrangement of Sirt2’s active
Figure 5 | SirReal inhibitors suppress Sirt2 activity via a unique mechanism. (a) Superposition of Sirt2–SirReal2–NAD$^+$ with ternary sirtuin complexes (Sir2Tm: PDB-ID 2H4F, aquamarine; Sirt3: PDB-ID 4FVT, raspberry) shows that NAD$^+$ of NCA ribose of NAD$^+$ (Sir2Tm) slightly enlarges the distance between the backbone carbonyl-O of Val233 to the N6 of Sirt2. Surprisingly, this rearrangement does not prevent Sirt2 from binding its acetyl-lysine peptide substrate, but deacetylation and the domain closure is blocked effectively.

Kinetic analyses of SirReal-mediated inhibition. In the course of the investigation SirReal-mediated inhibition, we also determined the crystal structure of another aminothiazole, termed SirReal2 (Fig. 4a), in complex with Sirt2 and a different acetyl-lysine peptide substrate. The latter is derived from ornithine transcarbamoylase (OTC, structure termed SirReal–OTC). SirReal1 has a benzyl instead of a naphthylmethyl substituent on the aminothiazole and is 26-fold less potent than SirReal2 in the same in vitro assay, but it retains high Sirt2 selectivity and shows similar behaviour in thermal stability assays (Supplementary Fig. 1b,c, Fig. 4b). Despite the presence of a different acetyl-lysine peptide, the structure of Sirt2–SirReal1–OTC bears a high resemblance to the Sirt2–SirReal2 complexes (r.m.s.d. (Cα atoms) = 0.44 Å to Sirt2–SirReal2–H3, 0.59 Å to Sirt2–SirReal2–NAD$^+$, Fig. 4c). SirReal1 also locks Sirt2 in the 

Site. It is more pronounced in comparison with the Sirt2 structure in complex with ADPR (PDB-ID 3ZGV, Fig. 3e) than with the structure of Sirt2–apo (PDB-ID 3ZGO, Fig. 3e).

A site of major rearrangement is the selectivity pocket of the hinge region, where the DMP ring of SirReal2 is bound (Fig. 3e). Here the loop region from Lys136–Phe143 is substantially shifted upwards with respect to the Sirt2–apo structure and forms a lid above the DMP moiety. These residues seem to be more flexible, indicated by high B-factors, in structures of the closed conformation such as the Sirt2–ADPR or Sirt2–ADPR–NCA complexes (Supplementary Fig. 2) than in structures of the open conformation.

Another site of SirReal2-induced rearrangement is observed in the acetyl-lysine-binding site. Here the side chains of the residues forming the highly hydrophobic acetyl-lysine tunnel, Tyr104, Phe119, Phe131, Phe234 and Phe235 are shifted (Fig. 3e). In particular, the side chains of Phe235 and Tyr104 that usually cap the acetyl-lysine are rotated towards the surface of Sirt2. Surprisingly, this rearrangement does not prevent Sirt2 from binding its acetyl-lysine peptide substrate, but deacetylation and the domain closure is blocked effectively.
open conformation and shows an almost identical interaction pattern as observed for SirReal2 (Fig. 4d).

To get insights into the inhibition mechanism, we first compared the structures of Sir2–SirReal complexes with the available sirtuin structures lacking inhibitors. For the cosubstrate NAD$^+$, the binding mode does not differ substantially. NAD$^+$ of Sirt2–SirReal2–NAD$^+$ also adopts a kinked conformation with a similar network of hydrophilic and hydrophobic interactions as observed for NAD$^+$ or Carba-NAD$^+$ in ternary sirtuin complexes (PDB-ID 2H4F$^\text{34}$, Sir2Tm-Ac-Lys-p53-peptide-NAD$^+$, PDB-ID 4FVT$^\text{23}$, Sir3-Carba-NAD$^+$-Ac-Lys-ACS-peptide, Fig. 5a).

As the main difference, the NCA ribose moiety of Carba-NAD$^+$ is rotated ~30° around its glycosidic bond compared with NAD$^+$ in Sir2–SirReal2–NAD$^+$ or Sir2Tm-Ac-Lys-p53-peptide-NAD$^+$. The binding mode of NAD$^+$ in Sir2–SirReal2–NAD$^+$ therefore shares a higher resemblance to the conformation of NAD$^+$ in the ternary complex with Sir2Tm.

The acetyl-lysine-binding modes in ternary sirtuin complexes and Sir2–SirReal2 structures on the other hand show substantial differences (Fig. 5b). In uninhibited sirtuin-acetyl-lysine-peptide structures, the acetyl-lysine-containing peptide binds in the cleft between the zinc-binding and NAD$^+$-binding domains, respectively, inserting its acetyl-lysine into a hydrophobic tunnel that is formed by several highly conserved phenylalanines. The binding of the acetyl-lysine is further stabilized by a hydrogen bond between the N$_\text{ε}$-H of the acetyl-lysine and the backbone carbonyl-O of a conserved valine. The hydrophobic acetyl-lysine–binding tunnel is not formed in all Sir2–SirReal2 structures, since Phe235, which usually caps the acetyl-lysine, is rotated ~90° towards the surface. Moreover, the bulky naphthyl moiety of SirReal2 forces the acetyl-lysine ~5 Å out of its physiological position, which can be seen in ternary complexes of sirtuins (Sir2Tm-Ac-Lys-p53-peptide-NAD$^+$, PDB-ID 2H4F, Sir3-Ac-Lys-ACS-peptide-Carba-NAD$^+$, PDB-ID 4FVT, Fig. 5b).

In the Sir2 complex with SirReal1, the acetyl-lysine-binding mode is different. The acetyl-lysine adopts an almost physiological position, as it is observed in the above-mentioned ternary sirtuin complexes. However, even in case of SirReal1, the benzyl moiety of SirReal1 shifts the acetyl-lysine of the OTC peptide.

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**Figure 6** | SirReal2 selectively inhibits Sirt2 via a Sirt2-specific amino acid network. (a) Structural sequence alignment of the Sirt1–6 deacetylase domain. The residues that presumably interact with SirReal2 are highlighted in yellow if they are equivalent to the residues of Sir2. If they differ from the residues of Sir2, they are highlighted in red. The structural sequence alignment was generated using T-Coffee$^\text{35}$ and slightly modified. (b–d) Surface representation of the binding pockets of SirReal2 in Sirt2–SirReal2–NAD$^+$ and in the homology models of Sirt1 (Sirt1-HM) and Sirt3 (Sirt3-HM). The residues that differ in the three isotypes are represented as sticks (Sirt2, slate blue; Sirt1, brown; Sirt3, turquoise). SirReal2 is shown as light pink sticks (Sirt2–SirReal2–NAD$^+$ complex with Sir2Tm).
images and uncropped blots are shown in the Supplementary Figs 8–10.

Structural aspects of isotype-selective inhibition

Portent Sirt2 inhibition.

The structural rigidity of Sirt2-bound SirReal2 are indispensable for formation of the intramolecular hydrogen bond and the resulting structural sequence alignment of the deacylase domain of Sirt1–6 most selective sirtuin inhibitors known to date.

SirReal2 does not alter p53-Lys382-acetylation after ultraviolet damage (n = 3). Raw immunofluorescent images and uncropped blots are shown in the Supplementary Figs 8–10.

To analyse the basis of this high isotype selectivity, we created a structural sequence alignment of the deacylase domain of Sirt1–6 and compared the crystal structure of the Sirt2–SirReal2 complex with available crystal structures of sirtuins in their open conformation (Fig. 6a, Supplementary Fig. 7a,b)35. Assuming that SirReal2 binds to the other sirtuin isotypes in a similar fashion as observed for Sirt2, Sirt4–6 exhibit major differences in their amino acid sequence. The structural differences are also very pronounced (Supplementary Fig. 7a) rationalizing the observed lacking in vitro inhibition of Sirt4–6 by SirReal2. Sirt1 and Sirt3, on the other hand, are phylogenetically more closely related to Sirt2 and show only minor sequence variations36. Their conformation is more similar to the Sirt2–SirReal2–NAD•+ complex than to the conformation of the isotypes Sirt5/6 (Supplementary Fig. 7b). But they still show major structural differences (r.m.s.d. (Cα atoms) = 1.6 Å). As it was not possible to dock SirReal2 in any of the available Sirt1 and Sirt3 X-ray crystal structures (Supplementary Methods), we wanted to probe whether Sirt1 and Sirt3 were able to adopt a similar conformation as observed in the Sirt2–SirReal2 structures that would allow binding of SirReal2. This would enable us to see whether the minor sequence variations within the deacylase domain of Sirt1–3 would have an influence on SirReal2 binding. Therefore, we generated homology models of Sirt1 (Sirt1-HM) and Sirt3 (Sirt3-HM) based on our Sirt2–SirReal2 structures (Supplementary Methods). Stereocchemical analyses as well as molecular dynamics simulations indicated high-quality model structures, and it was indeed possible to dock SirReal2 into these homology models (Supplementary Fig. 7c–h). However, the docking poses of SirReal2 in Sirt1-HM and Sirt3-HM gave less favourable docking scores compared with the requisite scores for the docking poses of SirReal2 in Sirt2–SirReal2 structures. Here the position and the conformation of SirReal2 were correctly predicted (Fig. 6b).

In case of Sirt1, residues Leu103, Ile118, Leu134, Leu138 and Leu206 of Sirt2 are substituted with Ile279, Met296, Phe312, Ile316 and Cys380 (Fig. 6c). Cys380 gives the hypothetical selectivity pocket of SirReal2 in Sirt1-HM a very different shape and changes its surface characteristics. The bulky substitution of the DMP moiety with a dimethylmercaptophenyl methylated amide leads to a submicromolar Sirt2 inhibition. The structural differences are also very pronounced (Supplementary Fig. 7a) rationalizing the observed lacking in vitro inhibition of Sirt4–6 by SirReal2. Sirt1 and Sirt3, on the other hand, are phylogenetically more closely related to Sirt2 and show only minor sequence variations36. Their conformation is more similar to the Sirt2–SirReal2–NAD•+ complex than to the conformation of the isotypes Sirt5/6 (Supplementary Fig. 7b). But they still show major structural differences (r.m.s.d. (Cα atoms) = 1.6 Å). As it was not possible to dock SirReal2 in any of the available Sirt1 and Sirt3 X-ray crystal structures (Supplementary Methods), we wanted to probe whether Sirt1 and Sirt3 were able to adopt a similar conformation as observed in the Sirt2–SirReal2 structures that would allow binding of SirReal2. This would enable us to see whether the minor sequence variations within the deacylase domain of Sirt1–3 would have an influence on SirReal2 binding. Therefore, we generated homology models of Sirt1 (Sirt1-HM) and Sirt3 (Sirt3-HM) based on our Sirt2–SirReal2 structures (Supplementary Methods). Stereocchemical analyses as well as molecular dynamics simulations indicated high-quality model structures, and it was indeed possible to dock SirReal2 into these homology models (Supplementary Fig. 7c–h). However, the docking poses of SirReal2 in Sirt1-HM and Sirt3-HM gave less favourable docking scores compared with the requisite scores for the docking poses of SirReal2 in Sirt2–SirReal2 structures. Here the position and the conformation of SirReal2 were correctly predicted (Fig. 6b).

In case of Sirt1, residues Leu103, Ile118, Leu134, Leu138 and Leu206 of Sirt2 are substituted with Ile279, Met296, Phe312, Ile316 and Cys380 (Fig. 6c). Cys380 gives the hypothetical selectivity pocket of SirReal2 in Sirt1-HM a very different shape and changes its surface characteristics. The bulky substitution of the DMP moiety with a dimethylmercaptophenyl methylated amide leads to a submicromolar Sirt2 inhibition. The structural differences are also very pronounced (Supplementary Fig. 7a) rationalizing the observed lacking in vitro inhibition of Sirt4–6 by SirReal2. Sirt1 and Sirt3, on the other hand, are phylogenetically more closely related to Sirt2 and show only minor sequence variations36. Their conformation is more similar to the Sirt2–SirReal2–NAD•+ complex than to the conformation of the isotypes Sirt5/6 (Supplementary Fig. 7b). But they still show major structural differences (r.m.s.d. (Cα atoms) = 1.6 Å). As it was not possible to dock SirReal2 in any of the available Sirt1 and Sirt3 X-ray crystal structures (Supplementary Methods), we wanted to probe whether Sirt1 and Sirt3 were able to adopt a similar conformation as observed in the Sirt2–SirReal2 structures that would allow binding of SirReal2. This would enable us to see whether the minor sequence variations within the deacylase domain of Sirt1–3 would have an influence on SirReal2 binding. Therefore, we generated homology models of Sirt1 (Sirt1-HM) and Sirt3 (Sirt3-HM) based on our Sirt2–SirReal2 structures (Supplementary Methods). Stereocchemical analyses as well as molecular dynamics simulations indicated high-quality model structures, and it was indeed possible to dock SirReal2 into these homology models (Supplementary Fig. 7c–h). However, the docking poses of SirReal2 in Sirt1-HM and Sirt3-HM gave less favourable docking scores compared with the requisite scores for the docking poses of SirReal2 in Sirt2–SirReal2 structures. Here the position and the conformation of SirReal2 were correctly predicted (Fig. 6b).

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SirReal6, resulted in a significant increase of (Supplementary Fig. 8a,b). Incubation with SirReal2, but not with concentrations and determined the level of cellular setting, we incubated HeLa cells with SirReal2 at various used as a tool to investigate the effects of Sirt2 inhibition in a in vivo measure for Sirt2 inhibition. Incubation with SirReal2 concentrations (Fig. 7b, Supplementary Fig. 8d,e). We also found that SirReal2 treatment did not alter cell cycle distribution, ruling out that the effect on BubR1 was indirect through induction of cell cycle changes (Supplementary Fig. 8f). To verify the observations from the western blot data, we also visualized the acetylation level by means of immunofluorescence microscopy. Again, the incubation with SirReal2 resulted in a partial increase of the acetylation of the microtubule network similar to the effects observed after treatment with the Sirt2-inhibitor AGK2 (Fig. 7a, Supplementary Fig. 9)39,40. SirReal6, on the other hand, does not alter microtubule acetylation. In addition, we analysed another Sirt2 target. Recently, we reported that the stability of spindle assembly checkpoint protein BubR1 is under control of Sirt2 (ref. 41). A decline in BubR1 over time has been linked to mammalian aging42. Deacetylation of Lys668 of BubR1 by Sirt2 inhibits the ubiquitination of BubR1 and its designation to the proteasome. Therefore, the abundance of BubR1 can be used as a functional measure for in vivo Sirt2 inhibition. Incubation with SirReal2 indeed significantly resulted in a dose-dependent depletion of BubR1, whereas SirReal5/6 did not influence BubR1 concentrations (Fig. 7b, Supplementary Fig. 8d,e). We also found that SirReal2 treatment did not alter cell cycle distribution, ruling out that the effect on BubR1 was indirect through induction of cell cycle changes (Supplementary Fig. 8f).

### Table 1 | Data collection and refinement statistics.

|                  | Sirt2–SirReal1–OTC* | Sirt2–SirReal2–H3* | Sirt2–SirReal2–NAD †* |
|------------------|---------------------|-------------------|-----------------------|
| **Data collection** |                     |                   |                       |
| Space group      | P2₁                 | P2₁               | I2                    |
| Cell dimensions (Å) | 36.21, 73.75, 55.86 | 35.99, 73.30, 55.29 | 83.7, 54.51, 96.69  |
| α, β, γ (°)      | 90, 94.71, 90       | 90, 95.23, 90     | 90, 114.8, 90        |
| Resolution (Å)†  | 44.43–1.45 (1.48–1.45) | 44.02–1.42 (1.44–1.42) | 48.28–1.88 (1.92–1.88) |
| Rmerge            | 0.126 (1.009)       | 0.060 (0.922)     | 0.068 (1.351)        |
| Rfree             | 0.054 (0.471)       | 0.035 (0.546)     | 0.029 (0.583)        |
| I/〈r〉           | 9.2 (1.7)           | 11.9 (1.5)        | 19.3 (1.5)           |
| Completeness (%)  | 99.9 (99.9)         | 99.6 (99.6)       | 100 (100)            |
| C1/C2             | 0.995 (0.549)       | 0.999 (0.534)     | 0.999 (0.585)        |
| Redundancy        | 6.5 (6.2)           | 3.7 (3.8)         | 6.6 (6.4)            |
| **Refinement**    |                     |                   |                       |
| Resolution (Å)    | 44.43–1.45          | 44.02–1.42        | 48.28–1.88           |
| No. of reflections | 334,389 (16,809)    | 199,366 (10,043)  | 213,173 (12,962)     |
| Rwork/Rfree (%)   | 26.0/28.2           | 18.1/18.8         | 20.2/24.7            |
| No. of atoms      |                     |                   |                       |
| Protein           | 2,251               | 2,406             | 2,350                |
| SirReal inhibitor | 25                  | 29                | 29                   |
| Ac-Lys peptide/NAD † | 32              | 12               | 44                   |
| Zn²⁺             | 1                   | 1                 | 1                    |
| Water             | 156                 | 242               | 99                   |
| B-factors (Å²)    |                     |                   |                       |
| Protein           | 17.3                | 21.1              | 38.4                 |
| SirReal inhibitor | 27.0                | 25.1              | 32.2                 |
| Ac-Lys peptide/NAD † | 28.9             | 48.3              | 41.8                 |
| Zn²⁺             | 11.7                | 14.8              | 30.7                 |
| Water             | 20.5                | 27.0              | 36.0                 |
| r.m.s. deviations | Bond lengths (Å)    |                   |                       |
|                  | 0.012               | 0.008             | 0.014                |
|                  | Bond angles (°)     |                   |                       |
|                  | 1.58                | 1.33              | 1.65                 |

*Each data set was obtained from one single crystal. Sirt2–SirReal1–OTC and Sirt2–SirReal2–H3 were collected at 1.0 Å at the Swiss Light Source (Villigen, Switzerland), Sirt2–SirReal2–NAD † was collected with an in-house X-ray source at 1.5418 Å.

†Values in parentheses are for highest-resolution shell.

Phe312 and Ile316 as well as Met296 and Ile279 also tighten the EC-site, resulting in an unfavourable orientation of the aminothiazole and naphthyl moieties in possible docking poses. In the case of Sirt3, the differences are mainly located at the selectivity pocket. Here Phe413, Thr171, Leu206 and Ile213 of Sirt2 are substituted by Tyr204, Gly232, Gly265 and Val272 (Fig. 6d). The less bulky Gly232, Gly265, Val272 of Sirt3 form a much wider and also more solvent-accessible selectivity pocket as compared with the Sirt2–SirReal2 structures. In contrast to the SirReal2-binding pockets of the homology models of Sirt1 and Sirt3, SirReal2 bound to Sirt2 can adopt a conformation that is in almost perfect complementarity with the protein, which is stabilized by the intramolecular hydrogen bond between the DMP substituent and the amide. This is not possible in Sirt1 and Sirt3 and also rationalizes the observed isotype selectivity.

**In vivo inhibition of Sirt2.** To validate that SirReal2 could be used as a tool to investigate the effects of Sirt2 inhibition in a cellular setting, we incubated HeLa cells with SirReal2 at various concentrations and determined the level of α-tubulin acetylation (Supplementary Fig. 8a,b). Incubation with SirReal2, but not with SirReal6, resulted in a significant increase of α-tubulin acetylation consistent with an in vivo inhibition of Sirt2 as shown previously35. These changes are not as pronounced when compared with the changes induced by the inhibition of the other main tubulin deacetylase KDAC6 (refs 38,39). KDAC6 activity is not affected in vitro in the presence of SirReal2 (Supplementary Fig. 8c). To verify the observations from the western blot data, we also visualized the acetylation level by means of immunofluorescence microscopy. Again, the incubation with SirReal2 resulted in a partial increase of the acetylation of the microtubule network similar to the effects observed after treatment with the Sirt2-inhibitor AGK2 (Fig. 7a, Supplementary Fig. 9)39,40. SirReal6, on the other hand, does not alter microtubule acetylation. In addition, we analysed another Sirt2 target. Recently, we reported that the stability of spindle assembly checkpoint protein BubR1 is under control of Sirt2 (ref. 41). A decline in BubR1 over time has been linked to mammalian aging42. Deacetylation of Lys668 of BubR1 by Sirt2 inhibits the ubiquitination of BubR1 and its designation to the proteasome. Therefore, the abundance of BubR1 can be used as a functional measure for in vivo Sirt2 inhibition. Incubation with SirReal2 indeed significantly resulted in a dose-dependent depletion of BubR1, whereas SirReal5/6 did not influence BubR1 concentrations (Fig. 7b, Supplementary Fig. 8d,e). We also found that SirReal2 treatment did not alter cell cycle distribution, ruling out that the effect on BubR1 was indirect through induction of cell cycle changes (Supplementary Fig. 8f). To determine if SirReal2 selectively inhibits Sirt2 in vivo, we assessed p53 acetylation following genotoxic stress (Fig. 7c, Supplementary Fig. 10a). Acetylation of p53 occurs in response to ultraviolet exposure to cells43 and this acetylation is regulated, in part, by the isotype Sirt1 (refs 44,45). On exposure to ultraviolet light, we detected an increase in acetylation of p53, which was further increased on treatment with the pan-sirtuin inhibitor...
NCA. However, we did not observe an increase in acetylation of p53 following treatment with SirReal2/5/6. Similarly, we also tested if SirReal2 could inhibit Sirt3 in cells by assessing mitochondrial protein acetylation. Sirt3 has previously been demonstrated to regulate global mitochondrial protein acetylation.46 Following treatment with SirReal2 or NCA as a positive control, we purified mitochondria and assessed protein acetylation by western blotting. We found that treatment with NCA leads to an increase in the acetylation of mitochondrial proteins, whereas treatment with SirReal2 did not, suggesting that SirReal2 is unable to regulate Sirt3 activity in cells (Supplementary Fig. 10b). These results confirm the in vitro observations and indicate that SirReal2 has a strong specificity towards Sirt2 in vitro when compared with the other members of Class-I sirtuins Sirt1 and Sirt3.

**Discussion**

There are many indications that sirtuins play an important role in neurodegeneration, cancer, bacterial infections and inflammation and that a modulation of Sirt2 activity could be a new strategy for pharmaceutical intervention. However, the physiological functions of Sirt2 are far from being completely understood and conclusive evidence for the suitability of Sirt2 as a pharmaceutical target is, at least in some cases, missing. To further explore Sirt2 function in a cellular environment, there is a definite need for selective and potent Sirt2 modulators.

So far, most sirtuin modulators lack either potency, selectivity or drug-like physicochemical properties. Recent screening campaigns have identified several potent and/or selective inhibitors15,24-26; however, with the exception of the macrocyclic peptide S2iL5, it is not clear how these inhibitors bind to Sirt2. And although several X-ray structures of sirtuins with inhibitors have been reported lately28,33,34-37, these structures do little to reveal a rationale for a Sirt2-selective inhibition.

With the identification of the SirReal inhibitors, we establish the structural basis for Sirt2-selective inhibition and report a new potent Sirt2-selective inhibitor scaffold. As noted above, the intramolecular hydrogen bond between the amide of the aminothiazole and a nitrogen atom of the DMP moiety gives the inhibitor a rigid form that can act as a molecular wedge locking the enzyme conformation with subsequent Sirt2 selectivity. Not only essential for the potency, the intramolecular bond also seems to play an important role for the compound’s Sirt2 selectivity, as it can only be formed when bound in perfect complementarity to the active site of Sirt2. This seems not to be possible if bind to Sirt1 or Sirt3.

Another important aspect alongside the internal hydrogen bond of SirReal-mediated Sirt2 inhibition is the exploitation of the selectivity pocket by SirReal inhibitors. The only other isotype-selective sirtuin inhibitors with known structures, CHIC-35 (Sirt1-selective)48, EX-527 (Sirt1-selective)33 and SRT1720 (Sirt3-selective)49 either bind to the EC-site and/or to the acetyl-lysine-binding site but neither the indole inhibitors nor SRT1720 or any of the inhibitors whose binding modes have been elucidated by means of X-ray crystallography occupy the selectivity pocket (Fig. 6). This pocket is formed by two loops that connect the Rossmann fold domain with the smaller zinc-binding domain. The residues that form this pocket significantly differ within the sirtuin family and targeting this pocket may present a new strategy for selective sirtuin inhibitor design. These particular findings would not have been discovered with the use of computational methods and the available Sirt2 structures.

In conclusion, with the identification of SirReal2, we provide an isotype-selective drug-like inhibitor with optimized potency and physicochemical properties in comparison with previously published Sirt2 inhibitors (Supplementary Table 1). We established valuable structural insights into selective Sirt2 inhibition and show that SirReal2 inhibits Sirt2 in vivo without affecting the activity of the other Class-I sirtuins Sirt1 and Sirt3. The observed selectivity towards Sirt2 may, in part, stem from a lack of penetration into the mitochondria but the cellular net result is as desired. SirReal2 may therefore be used for further cellular studies to probe Sirt2 biology. Our findings may constitute the basis for further selective sirtuin inhibitor development and provide a new tool for sirtuin biology.

**Methods**

**Cloning.** The gene sequences coding for human Sirt25-389 (Uniprot: Q96E86), human Sirt225-389, human Sirt3, 319-399, human Sirt4, 302 (Uniprot: Q9NXA8) and human Sirt513-308 (Uniprot: Q9N677) were purified as described before16,17. Sirt3118-395 (Uniprot: Q9NYQ7) was expressed and purified as described before14 with the exception that autoinduction with 0.2% (v/v) lactose in TB media was used for expression.

**Protein expression and purification.** Human Sirt1, 133-747 (Uniprot: Q96E86), human Sirt2, 25-389, human Sirt3, 319-399, human Sirt5, 302 (Uniprot: Q9NXA8) and human Sirt4, 308 (Uniprot: Q9N677) were purified as described before14,15. For this human Sirt1, 133-747, human Sirt2, 25-389, human Sirt3, 319-399 and human Sirt5, 302, an overnight digestion at 4°C, the digested protein was eluted with lysis buffer, concentrated and further purified using a Superdex 75 26/60 gel filtration column (GE Healthcare). The samples were then diluted to a peptide concentration of 50 mM Tris/HCl, 0.5 mM NaCl, 5% (v/v) glycerol, 5 mM β-mercaptoethanol, pH 8.0; Sirt2, 318-395, 500 mM HEPES, 500 mM NaCl, 5% (v/v) glycerol, 5 mM β-mercaptoethanol, pH 7.5) and lyzed using a microfluidizer (Microfluidics). After the removal of cell debris, the supernatant was applied to a HisTrapFF 5 ml column (GE Healthcare) and washed intensively before TEV protease ( excess) was applied directly on the column. After an overnight digestion at 4°C, the digested protein was eluted with lysis buffer, concentrated and further purified using a Superdex 75 26/60 gel filtration column (GE Healthcare). The samples were then diluted to a peptide concentration of 50 mM Tris/HCl, 100 mM NaCl, pH 8.0, Sirt2, 318-395, 25 mM HEPES, 200 mM NaCl, 5% (v/v) glycerol, 5 mM β-mercaptoethanol, pH 7.5). Sirtuin-containing fractions were collected and concentrated to 20 mg mL⁻¹. Additional purification steps were monitored using SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentration was determined by the Bradford assay (Roth).

**In vitro sirtuin assay.** Initial screens were conducted with a high-throughput fluorescence-based assay using the substrate ZMAL (Z-Lys(Acetyl)-AMC) that was synthesized according to published procedures26. For this human Sirt1, 133-747, human Sirt2, 25-389, human Sirt3, 319-399 and human Sirt5, 308, 50 mM Tris/HCl, 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂, pH 8.0, B-NAP + (final assay concentration 500 µM), the substrate ZMAL (final assay concentration 10.3 µM from a 12.6 mM stock solution in DMSO) and the respective inhibitor in DMSO at various concentrations or DMSO as a control (final DMSO concentration 5-20% (v/v)). The mixture was incubated at 37°C for 4 h, with agitation at 150 r.p.m. Deacetylation was then stopped by the addition of a solution containing NCA and trypsin (50 mM Tris/HCl, 100 mM NaCl, 6.7% (v/v) DMSO, trypsin 5.5 U ml⁻¹, 8 mM NCA, pH 8.0, 60 µl) and the mixture was then incubated for 20 min, 37°C, 150 r.p.m. Then the fluorescence intensity was measured in a microplate reader (BMG Polarstar, λex 390 nm, λem 460 nm). The amount of inhibition was determined with respect to the mixture with only DMSO. IC50 values were determined with Graphpad Prism software using a non-linear regression to fit the dose-response curve. SirReal and SirReal2 were also tested for Sirt3 inhibition using non-labelled acyl-lysine peptide substrates (Sirt5: Benzoyl-GLVLK(suclimycyl)EGGYV-NH2, 10 µM, Supplementary Methods). SirReal2 was also tested for Sirt4-6 inhibition using non-labelled acyl-lysine peptide substrates (Sirt6: Benzoyl-GLVLK(suclimycyl)EGGYV-NH2, 10 µM, Sirt7: Ac-EALPPR(Myristoyl)TG-NH2, 10 µM) The substrate was incubated (10 min, ~0.5 µM Sirt1/2/3/5/6, 500 µM β-NAP +, 5-20% (v/v) DMSO, 50 mM Tris/HCl, 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂, pH 8.0), stopped by the addition of trifluoroacetic acid (TFA, 10% (v/v), final concentration 1% (v/v)). The components of the stopped reaction mixture were separated by HPLC (Agilent 1100; Phenomenex reversed phase column Kinex RP18 2.7 µm, 30 x 3 mm) using a linear gradient of acetonitrile (20-80% (v/v) acetonitrile, 0.1% (v/v) TFA, 0.6 ml min⁻¹). Peaks of acetylated and deacetylated substrate were quantified by absorption at 280 nm. Sirt4-dependent deacetylation reactions were performed with an acetylated Ntt397-peptide (H-NITKLLK(Acetyl)AISPDK-NH2, 250 µM, Gl. Biochern., in 50 mM Tris/HCl, 150 mM NaCl, pH 7.5). Samples were taken between 0 and 45 min and reactions were stopped by mixing 1:1 with 6.5% (v/v) TFA. The samples were then diluted to a peptide concentration of 5 µM with 0.1% (v/v) formic acid and analysed on an EASY-nLCII connected to a LTQ mass...
spectrometer (Thermo Fisher Scientific). Peptides were separated by a linear gradient of acetonitrile (0–100% (v/v), 0.1% (v/v) TFA, 300 mM NaCl) on a reprosil C18 reversed-phase column. Peaks of deacetylated and deacetylated peptides were extracted using Skyline. A solution with DMSO was used as a negative control while a solution with the physiological inhibitor NCA served as a positive control (Fig. 1c). Owing to the lack of a suitable screening assay for human Sir2, we focused our studies on isoforms Sirt1–6. Fluor-de-Lys assays (Enzo Life Sciences) were conducted according to the manual using the HDAc80 Fluor-de-Lys deacetylate substrate (BML-K178-0005).

Protein crystallization. All crystallization trials were performed in 96-well plates (Intelli-Plate 96 Low Profile, Art Robbins Instruments) using an Oryx xenon pipetting robot (Douglas Instruments). Reservoir solutions were precooled to 4 °C and screens were then pipetted at 20 °C. For co-crystallization experiments with SirReal1 and SirReal2, a solution of the truncated Sirt2 (Sirt2-T256, 20 mg ml⁻¹ final concentration) was precipitated with a saturated aminothiazole solution (100 mM stock solution in DMSO, 1–2% (v/v) saturated aminothiazole solution (100 mM stock solution in DMSO, 1–2% (v/v) and 0.5 mM MgCl₂, 5% (v/v) DMSO, pH 8.0) was incubated (37 °C). The reaction was started by the addition of human Sirt2-S156–356 (2 μM), after 1–5 min with TFA (10% (v/v), final concentration 1% (v/v)) and analysed by HPLC as described above. The peak areas were integrated and converted to initial velocities calculated from the peak areas as the fraction of deacetylated peptide from total peptide. From this, reaction rates in μM min⁻¹ were obtained by linear regression, while k_on and k_off were obtained directly from Michaelis–Menten plots using Graphpad Prism software. β-NAD⁺ (500 μM) was used for the determination of the kinetic parameters for the peptide substrate, 100 μM of peptide substrate was used for the determination of the kinetic parameters for β-NAD⁺.

Thermal shift assays. Human Sirt2-S156–356 (0.2 mg ml⁻¹ final concentration) was mixed with or without binding buffer (25 mM Tris/HCl, 150 mM NaCl, 5% (v/v) DMSO, 1:400 Sypro Orange, pH 8.0) in absence or presence of β-NAD⁺ (100 mM stock solution in 25 mM Tris/HCl, 150 mM NaCl, pH 8.0, final assay concentration 5 mM) or acetylated H3-peptide (100 mM stock solution in 25 mM HEPES, 200 mM NaCl, 5% (v/v) glycerol, pH 7.5, final assay concentration 5 mM). Thermal stability of the complexes was monitored during a temperature ramp from 25–95 °C (1 °C min⁻¹) using a Bio-Rad iCycler iQ5 (4titude, FrameStar 96-well plates, 4t-0771, 4titude qPCR Seal, 4t-0560). Melting temperatures were determined according to published procedures using Graphpad Prism software.

Cell cultivation. HEK cells (DSMZ accession no. 057) and U2OS cells (ATCC accession no. HTB-96) were grown in Dulbecco’s modified Eagle’s medium (PAA) containing 10% (v/v) fetal calf serum (FCS, PAA), 1% (v/v) penicillin (PAA), 1% (v/v) streptomycin (PAA), 1% (v/v) -glutamine (PAA) at 37 °C in a 5% (v/v) CO₂ atmosphere.

Tubulin acetylation. HEK cells were plated in petri dishes (5 cm, PAA), incubated overnight to a confluency of 30–40% and then treated with SirReal2 dissolved in RPMI1640 medium supplemented with fresh 20% (v/v) FCS (PAA), 1% (v/v) penicillin (PAA), 1% (v/v) streptomycin (PAA), 1% (v/v) -glutamine (PAA), 1% (v/v) DMSO for 5 h at various concentrations. Cells were then washed with prewarmed PBS (2 ml), lysed in SDS-PAGE sample buffer (70 μl, 50 mM Tris/HCl, 0.5 mM EDTA, 1% Complete Protease Inhibitors (Sigma-Aldrich), 2% (v/v) IGEPAL (Sigma-Aldrich), 2% (v/v) SDS, 10% (v/v) glycerol, 50 mM NCA (Sigma-Aldrich), 3.3 mM trichostatin A (Sigma-Aldrich), 50 mM DTT, 0.01% (w/v) bromophenol blue, pH 6.8) and sonicated for 1 min. Cell samples were separated using SDS-PAGE (12.5% (w/v) polyacrylamide), transferred to an nitrocellulose membrane (Bio-Rad), blocked with non-fat dry milk (Roth, 5% (v/v), TBS, 0.1% (v/v) Tween 20) and probed with an anti-acetyl-α-tubulin antibody (1:10,000, Sigma-Aldrich, T6793) and an anti-GAPDH antibody (1:10,000-1:50,000, Sigma-Aldrich, G9545) as a loading control (Fusion SL, quelab). An uncropped blot is shown in Supplementary Fig. 8.

Abundance of BubR1. HEK cells plated in six-well plates were treated with SirReal2 dissolved in FCS (1% (v/v) DMSO, 16 h) at various concentrations. Cells were washed with PBS, lysed in IPI5 (50 mM Tris/HCl, 150 mM NaCl, 0.5 mM EDTA, 0.3% (v/v) NP-40, pH 7.5, supplemented with Complete protease inhibitors (Roche)). Samples were pelleted and resuspended in 1 × SDS-PAGE sample buffer (70 μl, 5% (v/v) DMSO, 1.5% (v/v) pH 8.0, sodium dodecyl sulphate (2% (w/v)) and sonicated for 1 min. Cell samples were separated using SDS-PAGE (10% (w/v) polyacrylamide), transferred to a nitrocellulose membrane (Bio-Rad), blocked with non-fat dry milk (Roth, 5% (v/v), TBS, 0.1% (v/v) Tween 20) and probed with the anti-BubR1 (1:5,000, BD Biosciences, 612020) and anti-tubulin (1:5,000, Sigma-Aldrich, T5168) as a loading control. Uncropped blots are shown in the Supplementary Fig. 8.

Immunocytochemistry. HEK cells that were incubated with SirReal2 (20 and 50 μM), SirReal6 (50 μM), AGK2 (Sigma-Aldrich, 20 μM) or DMSO as a control in Dulbecco’s modified Eagle’s medium supplemented with 10% FCS, antibiotics and DMSO (1% (v/v)) for 4 h, were fixed with ice-cold methanol (10 min), washed with PBS and blocked with PBS supplemented with 0.1% (v/v) Triton-X 100 and 0.5% (v/v) FCS (30 min). Cells were then stained with an anti-acetyl-α-tubulin antibody (Sigma-Aldrich, T6793) and then probed with a secondary Alexa 488 conjugated anti-mouse-antibody (Invitrogen). Nuclei were counterstained with DAPI.
p53 Acetylation. U2OS cells (ATCC accession no. HTB-96) were seeded and cultured until they reached 90% confluency. Cells were then pretreated for 1 h with NCA (Sigma-Aldrich), SirReal2, SirReal5 or SirReal6 at the indicated concentrations, and then subsequently exposed to 20 cm2 of ultraviolet light. Cells were incubated for an additional 6 h in the presence of the inhibitor and then lysed in IPLS (50 mM Tris/HCl, 150 mM NaCl, 0.5 mM EDTA; 0.5% (v/v) NP-40: 1 × Complete Protease Inhibitors (Roche), pH 8.0) and resuspended in 1 × Laemmli Buffer. Samples were then separated on SDS–PAGE, transferred to a nitrocellulose membrane (Bio-Rad), blocked with non-fat dry milk (Bov., 5% (w/v), TBS, 0.1% (v/v) Tween 20) and probed with anti-acetyl-p53 K382 (Cell Signaling, #2522), anti-p53 DO.1 (Santa Cruz Biotechnology, sc-126) and anti-vinculin (Cell Signaling, #4400) as a loading control. Uncropped blots are shown in the Supplementary Fig. 10.

References

1. Feldman, J. L., Baeza, J. & Denu, J. M. Activation of the protein deacetylase DAPI (4-

2. Jiang, H. et al. SIRT6 regulates TNF-α secretion through hydrolysis of long-chain fatty acyl lysine. Nature 496, 110–113 (2013).

3. Du, J. et al. Sir1 is a NAD-dependent protein lysine demalonylase and desuccinylase. Science 334, 806–809 (2011).

4. Du, J., Jiang, H. & Lin, H. Investigating the ADP-ribosyltransferase activity of sirtuins with NAD analogues and NADP. Biochemistry 49, 2878–2890 (2010).

5. Vaquero, A., Sternglanz, R. & Reinberg, D. NAD + -dependent deacetylation in bacterial infection. PLoS Pathog. 6, e1001135 (2010).

6. North, B. J., Marshall, B. L., Borra, M. T., Denu, J. M. & Verdin, E. The human Sirt5 is a NAD-dependent lysine desuccinylase. Proc. Natl Acad. Sci. USA 108, 852–857 (2011).

7. Chopra, V. et al. Structure and functional analysis of the human Sir2 and its ADP-ribosyl complex. J. Biol. Chem. 286, 31350–31356 (2011).

8. Hoffmann, G., Breitenbuecher, F., Schuler, M. & Ehrenhofer-Murray, A. E. A novel sirtuin 2 inhibitor (SirT2) Inhibitor with p33-dependent pro-apoptotic activity in non-small cell lung cancer. J. Biol. Chem. 289, 5208–5216 (2014).

9. Schlicker, C., Boanca, G., Lakshminaraisingham, M. & Steegborn, C. Structure-based development of novel sirtuin inhibitors. Aging 3, 852–872 (2011).
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Author contributions
T.R., W.S., O.E. and M.J. designed the study and wrote the paper. T.R. performed the crystallization experiments, collected the data and analysed all the data. S.G. analysed the structural data. M. Schiedel synthesized SirReal inhibitors. C.R. performed the kinetic analysis. T.R., C.R., M.P., M. Schiedel and M. Schutkowski analysed in vitro data. B.J.N., T.R., K.S., K.L.L., A.L., J. Oláh, J. Ovádi and K.L.L. performed the cellular biology. T.R., B.J.N., A.L., J. Ovádi and D.A.S. analysed the cellular data. B.K. and W.S. performed computational analysis. All authors discussed and commented on the manuscript.

Additional information
Accession codes: Coordinates and structure factors of the Sirt2-SirReal2-NAD+ complex (4RMI) and the Sirt2-ADPR-NCA (4RMJ) complex have been deposited in the Protein Data Bank under the above-mentioned accession codes.

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

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