Cite this: *Med. Chem. Commun.*, 2019, 10, 1550

Received 8th May 2019,
Accepted 21st June 2019

DOI: 10.1039/c9md00263d

rsc.li/medchemcomm

Emerging modes-of-action in drug discovery

Eric Valeur, ^{*a} Frank Narjes, ^b Christian Ottmann ^{cd} and Alleyn T. Plowright^e

An increasing focus on complex biology to cure diseases rather than merely treat symptoms has transformed how drug discovery can be approached. Instead of activating or blocking protein function, a growing repertoire of drug modalities can be leveraged or engineered to hijack cellular processes, such as translational regulation or degradation mechanisms. Drug hunters can therefore access a wider arsenal of modes-of-action to modulate biological processes and this review summarises these emerging strategies by highlighting the most representative examples of these approaches.

1. Introduction

For decades, drug discovery has been centred around directly modulating protein functions. Derived from the lock and key

concept of small molecules fitting into enzyme or receptor pockets to activate or inhibit their function, many marketed drugs are living examples of the success of this strategy. In the 2000s, the limitation of small molecules for binding larger surface areas of some proteins has led to the rise of biologics, most notably antibodies, but still with the same paradigm of directly activating or blocking function.

Despite these successes, the necessity to discover novel biology to more precisely modulate and moreover cure diseases is changing the target landscape.¹ In this respect, a target no longer necessarily represents a protein, and modulation at the RNA and DNA level is becoming a reality. Genome editing technologies offer the promise of correcting genetic defects at the DNA level.^{2,3} Alternative strategies to modulate proteins, for example by preventing translation through degradation of mRNA, is one example of a different mode-of-

^a Medicinal Chemistry, Research and Early Development, Cardiovascular, Renal & Metabolism, BioPharmaceuticals R&D, AstraZeneca, Gothenburg, 43183 Mölndal, Sweden. E-mail: evaleur@yahoo.fr

^b Medicinal Chemistry, Research and Early Development, Respiratory, Inflammation and Autoimmune (RIA), BioPharmaceuticals R&D, AstraZeneca, Gothenburg, 43183 Mölndal, Sweden

^c Department of Biomedical Engineering and Institute for Complex Molecular Systems, Technische Universiteit Eindhoven, Den Dolech 2, 5612, AZEindhoven, the Netherlands

^d Department of Chemistry, University of Duisburg-Essen, Universitätsstraße 7, 45117, Essen, Germany

^e Integrated Drug Discovery, Sanofi-Aventis Deutschland GmbH, Industriepark Höchst, D-65926 Frankfurt am Main, Germany

Eric Valeur

Eric Valeur has over 15 years drug discovery experience gained at Merck, Novartis, and most recently AstraZeneca. He has led medicinal chemistry teams across several therapeutic areas and across modalities, including small molecules, macrocycles, peptides, oligonucleotides, drug conjugates, and PROTACs, delivering clinical candidates and novel technologies. He is passionate about challenging targets and disruptive drug discovery, in

particular in combination with novel biology. Eric is coauthor/coinventor of over 50 publications, patents, posters, and oral presentations and holds a Ph.D. from the University of Edinburgh (under supervision of Prof. Mark Bradley) and an MBA from Imperial College London.

Frank Narjes

Dr. Frank Narjes is currently Senior Principal Scientist in the Department of Medicinal Chemistry at AstraZeneca, Sweden and Project Leader for early targets in the Respiratory, Inflammation and Autoimmunity area. His current work in AstraZeneca focusses on 'new modalities' such as bicyclic peptides and heterobifunctional degraders. Prior to joining AstraZeneca, he was employed at Merck, Sharp & Dohme in Rome, where he explored protease and polymerase inhibitors for the treatment of Hepatitis C virus infection. Frank studied chemistry at the University of Hamburg, followed by postdoctoral studies at UC Irvine.

action (MOA) taken to the market.⁴ Several oligonucleotide entities are indeed either approved or undergoing development for a range of diseases including hypercholesteremia, cancer, diabetes, and Duchenne muscular dystrophy.

In addition, non-coding RNAs (ncRNAs), which represent about 80% of the genome, are increasingly associated with disease phenotypes and may foster a new range of targets to modulate protein functions through novel regulation mechanisms. Observing how nature regulates biological entities and processes provides a wide range of opportunities to leverage these in a therapeutically-meaningful manner. For example, a protein without the appropriate function, *e.g.* a misfolded protein, may in some cases be depleted through degradation. This can be achieved first through ubiquitination, to then direct the protein to the proteasome for degradation. Recapitulating this principle with a drug modality allows the exploitation and redirection of a natural process towards other biological entities of therapeutic relevance and has given birth to the field of targeted protein degradation.⁵

This opportunity to hijack natural biological processes as a source of inspiration for leveraging or creating novel MOAs dramatically increases the range of options to modulate biological functions across therapeutic areas. Consequently, this expanding number of potential MOAs result in a vast opportunity to develop probes and drugs towards novel targets.^{1,6} In this respect, this review aims at providing a brief overview of emerging MOAs that can be taken advantage of by contemporary medicinal chemists, and is restricted to mechanisms that have, to date, not reached the market. The opportunities include stabilising protein–protein interactions, promoting protein degradation and a range of MOAs aimed at upregulating or downregulating protein levels by direct or indirect modulation of RNAs (Fig. 1).

2. Stabilising protein–protein interactions

Targeted stabilisation of protein–protein interactions (PPIs) is an underexplored concept in drug discovery, despite numerous examples from natural products and synthetic molecules advocating for this principal strategy.^{7–9} For example, the immunosuppressants FK506 and rapamycin stabilise the complex of their primary binding protein – FKBP12 – to the phosphatase calcineurin (FK506) and the protein kinase mTOR (rapamycin). These two enzymes are thus ‘glued’ to FKBP12 by the natural products which leads to their inactivation that in turn explains their immunosuppressant activity.^{10,11} Other natural products that act as PPI stabilisers include brefeldin A,¹² forskolin,¹³ and the phytohormones auxin,¹⁴ and jasmonate.¹⁵

Notably, synthetic drugs that operate by stabilising PPIs have almost exclusively been recognised as PPI stabilisers after their therapeutic relevance was shown and in many cases even long after FDA approval. A prominent example is thalidomide and its derivatives. More than 30 years after market withdrawal in 1961, thalidomide was reintroduced as an immunomodulatory drug in 1998 for the treatment of erythema nodosum leprosum and in 2005 for plasma cell myeloma.¹⁶ In the same year, a derivative of thalidomide, lenalidomide, was approved as a therapy for myelodysplastic syndrome.¹⁷ Although these approvals were a game-changer for the treatment of these malignancies, it took another ten years before the MOA of these molecules was elucidated in full detail by the crystal structure showing how lenalidomide initiates and stabilises the binding of oncogenic proteins such as CK1 α to the ubiquitin ligase CRL4^{CRBN}.¹⁸ In this case, lenalidomide acts as a ‘molecular glue’ that hijacks the ubiquitin ligase for the targeted degradation of a neo-substrate protein.¹⁹

Christian Ottmann

Christian Ottmann is Associate Professor at Eindhoven University of Technology, The Netherlands. He works on stabilization of 14-3-3 adapter protein PPIs by small molecules. He is coordinator of the FP7 Industry-Academia Partnership and Pathways (IAPP) 14-3-3STABS and the Horizon2020 European Training Network (ETN) TASPPI. Before taking up his current position in Eindhoven he was a group leader at the Chemical Ge-

nomics Centre (CGC) in Dortmund, Germany. In 2012 he was recipient of the Innovation Award in Medicinal/Pharmaceutical Chemistry of the GDCh/DPhG and in 2013 of the Young Chemical Biology Award of the International Chemical Biology Society (ICBS).

Alleyn Plowright

Dr. Alleyn Plowright obtained his PhD in organic chemistry with Professor Gerald Pattenden at the University of Nottingham in 1999, and continued with postdoctoral studies with Professor Andrew Myers at Harvard University. In 2002, Alleyn joined AstraZeneca in the UK as a Medicinal Chemist before moving to AstraZeneca Sweden in 2008. In 2017 Alleyn moved to Sanofi as Head Integrated Drug Discovery Germany leading a

cross-disciplinary research unit driving projects from target validation through to pre-clinical development. His current research interests include Drug Design, Phenotypic Drug Discovery and the use of diverse chemical modalities to treat metabolic, cardiovascular, immunological and rare diseases.

Fig. 1 Overview of classical (agonism/antagonism) and emerging modes of action.

Lenalidomide binds to a composite pocket created by both $\text{CLR4}^{\text{CRBN}}$ and $\text{CK1}\alpha$ and is almost entirely buried between the two proteins (Fig. 2A). The glutarimide moiety is accommodated by a hydrophobic pocket formed by three tryptophan residues (W380, W386, W400) and one phenylalanine (F402) from $\text{CLR4}^{\text{CRBN}}$. Further hydrophobic contacts are provided by $\text{CK1}\alpha$ Ile35 with C5 and C6 of the lenalidomide phthalimide ring (Fig. 2B). These van-der-Waals interactions are complemented by a number of polar contacts formed between lenalidomide and both elements from $\text{CLR4}^{\text{CRBN}}$ and $\text{CK1}\alpha$, for example between side-chain nitrogens of H378 and W380 and the C2 carbonyl of the drug (Fig. 2C). It is the combination of simultaneous contacts to both proteins that makes lenalidomide an effective stabiliser or inducer of the $\text{CLR4}^{\text{CRBN}}/\text{CK1}\alpha$ protein–protein interaction, which ultimately leads to degradation of $\text{CK1}\alpha$.

Another example is the stabilisation of transthyretin (TTR) tetramers. Amyloid diseases are characterised by the deposition of protein aggregates known as amyloid fibrils.²⁰ In this context, transthyretin amyloidosis (ATTR) is believed to be caused by the aggregation of TTR.²¹ Under healthy, physiological conditions TTR exists as a tetramer, but disassembly

into smaller oligomers, misassembly and misfolding have been identified as possible causes of TTR aggregation and ATTR development.²² Based on the discovery that the natural thyroid hormone (S)-thyroxine (T4) is an inhibitor of TTR aggregation and a stabiliser of the TTR tetramer, small molecule inducing stabilisation of the tetramer has been used as a strategy.^{23,24} One specific compound, tafamidis, approved in Europe and Japan,²⁵ stabilises the TTR tetramer by binding to the interface of two component proteins as shown by X-ray crystallography,²⁶ and thus inhibits the formation of fibrils. More importantly, it also stabilises two clinically relevant mutant forms, making it a broadly applicable drug in early stage ATTR.²⁶ Additional efforts in this field have led to the discovery to tolpacone, which has a higher TTR tetramer stabilising potency, likely due to a better fit into the interface pocket, as shown by X-ray crystallography.²⁷

Other examples of marketed drugs which have been subsequently shown to stabilise protein–protein interactions include dexrazoxane and ifenprodil. Dexrazoxane binds to topoisomerase II (topo II) and locks the dimeric enzyme in an inactive closed clamp state by binding to a symmetric pocket formed by the two topo II monomers.²⁸ Ifenprodil binds to the interface of the GluN1 and GluN2 ion channels, and exerts its activity by stabilising the dimer and inhibiting channel function.²⁹

Since the retrospective discovery of some marketed drugs having PPI stabilisation as MOA, efforts are being directed to target this MOA as a primary strategy. In this respect, 14-3-3 PPI stabilisers represent an illustrative category. There is increasing evidence of association between abnormal expression of 14-3-3 and diseases, including in cancer where overexpression and survival are negatively correlated, which positions these proteins as potential therapeutic targets. Indeed, 14-3-3 proteins are one of the most connected proteins

Fig. 2 Lenalidomide stabilising the $\text{CLR4}^{\text{CRBN}}/\text{CK1}\alpha$ complex. A. Overview of the $\text{CLR4}^{\text{CRBN}}/\text{CK1}\alpha$ complex depicting the C-terminal domain (CTD) of $\text{CLR4}^{\text{CRBN}}$ (cyan) and $\text{CK1}\alpha$ and the composite binding pocket of lenalidomide (insert, yellow sticks). B. Hydrophobic interactions between lenalidomide and highlighted residues from $\text{CLR4}^{\text{CRBN}}$ (cyan sticks) and $\text{CK1}\alpha$ (olive sticks). C. Polar contacts of lenalidomide binding to both proteins. (PDB ID 5FQD).

in the human interactome with several hundred experimentally validated partner proteins,³⁰ and around 2000 proteins having shown binding in proteomic studies.³¹ The promiscuous nature of the 14-3-3 proteins may prove an advantage. For example, many pro-proliferative pathways are regulated by 14-3-3 proteins at multiple nodes. In the Ras–Raf–MAPK pathway 14-3-3 protein binds to and inhibits the activity and signal transduction of Sos1,^{32,33} KSR,³⁴ and all three isoforms of the Raf kinases.^{35–38} Simultaneous stabilisation of these negative regulations by 14-3-3 protein could potentially attenuate pro-proliferative signalling in a way that would make it hard for a cancer cell to develop countermeasures based on concurrent mutations in multiple proteins.

Furthermore, the rich diversity of 14-3-3 interacting proteins ranging from ion channels (*e.g.* TASK-1 (ref. 39)) and protein kinases (*e.g.* LRRK2 (ref. 40 and 41)) to transcription factors such as Myc,⁴² defines targeted and specific stabilisation of 14-3-3 PPIs as an attractive MOA for the development of novel bioactive compounds. In principle, assuming that specificity demands are fulfilled, a considerable number of human diseases could be addressed with 14-3-3 PPI stabilisers. Finally, the involvement with notoriously hard-to-drug intrinsically disordered proteins (IDPs) such as tau,⁴³ α -synuclein,⁴⁴ huntingtin,⁴⁵ or ataxin-1,⁴⁶ may present 14-3-3 PPIs as highly relevant targets in the field of neurodegeneration. These PPIs involve a disorder-to-order transition creating a defined joint 14-3-3/IDP interface with 'ligandable' pockets and targeting these interfaces may hold the key for a successful small-molecule modulation of IDP functions.

In recent years, a number of small molecules have been reported as modulators of 14-3-3 PPIs.⁴⁷ The first molecules discovered to target 14-3-3 proteins were natural products of the diterpene glycoside class of fusicoccanes.^{48,49} For example, fusicoccin A (FC-A) has been shown to bind to the interface of 14-3-3 and ER α ,⁵⁰ CFTR,⁴⁰ and p53,⁵¹ leading to the stabilisation of these target complexes, as well as binding to a number of 14-3-3 complexes involved in axon regeneration.^{52,53} The FC-A-related compound cotylenin A (CN-A) enhances binding of 14-3-3 to the N-terminus of C-Raf,⁵⁴ and the semi-synthetic FC derivatives ISIR-005 and FC-THF bind to and stabilise the 14-3-3 complexes with Gab2,⁵⁵ and Task3.⁵⁶ Furthermore, FC-Nac, a ten fold more potent derivative of FC-J, was recently published.⁵⁷ Since fusicoccanes are challenging molecules for medicinal chemistry optimisation due to their structural complexity, fragment-based approaches have recently been explored to identify ligands which bind to these complexes.^{58–60} Here, the composite binding pocket formed by the interaction of an ER α -derived peptide and 14-3-3 σ that accommodates FC-A could be populated with fragments employing the technique of 'tethering'.⁶⁰

In addition to natural products, supramolecular ligands have been shown to modulate 14-3-3 PPIs. The molecular tweezer CLR01 binds to a lysine residue located near the central, peptide-binding channel of the adapter protein and thereby inhibits 14-3-3's interaction with partner proteins

such as ExoS and C-Raf (Fig. 3).⁶¹ Interestingly, the same molecule (CLR01) does not inhibit but rather stabilises the binding of a Cdc25C-derived peptide with 14-3-3.⁶² This differential activity is explained by specific interactions consisting of CLR01 binding to a specific arginine residue of the Cdc25C peptide and by the hydrophobic outer surface of the molecule which 'glues' the peptide more strongly into the 14-3-3 binding channel.

Small-molecule PPI stabilisation features a number of unique advantages for drug discovery including the non-competitive nature of the ligands and the higher potential to achieve specificity as a result of binding to composite surfaces. Importantly, such binding sites are enriched in regulatory protein complexes because of the imperfect shape complementarity in protein–protein surfaces.⁶³ It also offers novel opportunities to address undruggable targets, as illustrated recently with the identification of stabilisers of the Max transcription factor homodimer, resulting in reduced levels of the Myc oncogene.⁶⁴

3. Downregulation of protein levels through protein degradation

Degradation of proteins induced by small molecules has become one of the most exciting emerging MOAs in drug discovery.^{65–67} The high interest is derived from several benefits associated with this mechanism compared to the traditional inhibition model, which is typically characterised by occupancy of an allosteric or active site from a receptor or enzyme. Protein degradation offers the premise of not only abolishing protein activity, usually achieved with classical antagonism, but also its other functions, such as scaffolding or the regulation of PPIs. Therefore, degradation offers a more diverse and complete target pharmacology profile than inhibition.⁶⁸

In addition, the pharmacodynamic effect of a protein degrader does not only rely on its exposure at the site of action but also on the resynthesis rate of the target protein, offering the possibility of extended duration of action. Finally, in the context of oncology and infectious diseases, protein degradation may reduce the risk of resistance.

Small molecule-based protein degraders can therefore mimic the response obtained by methods such as RNA interference or antisense oligonucleotides and present the possibility to combine the efficacy of gene-silencing methods with the advantages of small molecule drugs, such as cell permeability, oral bioavailability and temporal control over the dosing regimen.^{5,69} Protein degradation can be achieved by different means including direct degradation inducers, hydrophobic tagging and recruiters of E3 ligases (Fig. 4).

3.1. Degradation induced by small molecule binders

While the field of protein degradation has been popularised with bivalent molecules, several small molecules have been shown to directly induce degradation of their target. A significant example is fulvestrant (Fig. 5), a selective oestrogen

Fig. 3 Examples of 14-3-3 PPI stabilisation with different modalities: Cotylenin A (PDB ID: 4IHL), Fusicoccin, Pyrrolidone 1 (3M51), Tweezer, fragments (6HHP), and the molecular tweezer CLR01 (5M36). 14-3-3 is shown as white surface and the different partner protein peptides as sticks in different colour.

receptor down regulator (SERD), approved for oestrogen receptor (ER) positive breast cancer. Initially developed as a conventional oestrogen receptor antagonist, mechanistic in-

vestigations revealed that the bulky side-chain of fulvestrant induces a more profound change of the receptor's co-factor recruitment site around helix 12, which is responsible for co-

Fig. 4 Examples of degradation mechanisms.

activator and co-repressor binding. This exposes a larger hydrophobic surface compared to other ER antagonists and leads to ubiquitination and subsequent degradation of ER.⁷⁰ Recent efforts in discovering orally bioavailable SERD's have reinforced the notion that modulation of the conformation of helix 12 is linked to ER degradation.^{71,72}

Interestingly, a surprising number of ATP-competitive kinase inhibitors have been linked to protein degradation through a number of mechanisms.⁷³ These include internalisation and lysosomal degradation as described for c-KIT upon imatinib binding, transcriptional modulation of mRNA levels by the pan-JAK inhibitor PF-956980, or disruption of the interaction of the kinase domain with the Hsp90 chaperone,⁷³ as exemplified by the irreversible tyrosine kinase inhibitor ligand CI-1033 which induces ubiquitination, endocytosis and degradation of ErbB-2.⁷⁴

Another example of protein destabilisers is the inhibitors of the BTB domain of the transcription factor BCL6, an oncogenic driver in diffuse large B cell lymphoma (DLBCL). Minor structural changes convert an inhibitor to a degrader, which acts *via* induction of ubiquitination (Fig. 5). Notably, the degraders exhibit stronger anti-proliferative effects and a greater induction of expression of BCL6-repressed genes compared to the structurally related inhibitor.⁷⁵

Other examples of small molecule degraders include the androgen receptor antagonist bicalutamide,⁷⁶ and BHM-21, a ribosomal DNA binder, which inhibits transcription by polymerase I and leads to degradation of its catalytic subunit RPA194.⁷⁷

Recently, another category of monovalent degradation inducers was developed by identifying molecules capable of stabilising the naturally occurring PPI between the protein of interest and an E3 ligase.⁷⁸ In this case, the molecule leverages the molecular 'glue' concept described in section 1 to stabilise the interaction between β -catenin and the β -TrCP E3 ligase. Degradation based on small molecule inducers may offer a significant advantage in terms of ADME properties compared to the other types of degraders discussed below. However, designing such molecules at first intent is not obvious.

Fig. 5 Representative examples of monovalent protein degradation inducers.

3.2. Hydrophobic tagging

In principle, hydrophobic tagging mimics the mechanism of protein destabilisation described above by appending a hydrophobic moiety to a protein ligand, and hence to the protein upon binding of the molecule.⁷⁹ Protein tagging signals to the cell that the protein is misfolded, initiating the degradation process. The method was initially developed using HaloTag fusion proteins,⁸⁰ and applied to the pseudokinase HER3. Conjugation of a hydrophobic adamantyl moiety to the covalent inhibitor TX-85-1 led to the bivalent ligand TX2-121-1 (Fig. 6). Enhanced inhibition of HER3-dependent signalling in a PC9 lung carcinoma cell line was observed, which was due to induction of partial degradation of HER3 *via* the proteasome. The compound also interfered with the ability of HER3 to heterodimerise with either HER2 or c-Met.⁸¹ This strategy has also been applied to ligands of the androgen receptor by converting RU59063, a known agonist, to a degrader through conjugation to the adamantyl moiety. The construct also retained activity in cell lines resistant to current standard of care.⁸²

The approach can be applied to targets lacking small molecule binders. For example, a construct, consisting of a cell-penetrating peptide and a TAR DNA binding protein 43 (TDP-43) recognition peptide, connected *via* a linker to two adamantyl molecules, achieved degradation of the target protein in cells, and in a transgenic *Drosophila* model of amyotrophic lateral sclerosis.⁸³

Using a similar approach, a construct targeting the tau protein, involved in the formation of neurofibrillary tangles in Alzheimer's disease, was able to reduce intracellular protein levels, which was shown to depend on the proteasome and the hydrophobic tag. Intravenous dosing of the compound for eight days in a mouse model of Alzheimer's disease reduced tau levels in mouse brain.⁸⁴

Hydrophobic tagging can also be achieved with different tags. For example, arginine protected at the guanidino group with tertiary-butyl carbamate groups (Boc₃Arg) can be linked to small molecules and has been shown to induce degradation of targets such as glutathione transferase and bacterial

Fig. 6 Representative examples of hydrophobic tagging.

dihydrofolate reductase (Fig. 6). Interestingly, this method does not seem to require ubiquitination of the target protein. Instead, it was shown that the tag directly localises the protein to the 20S proteasome for degradation.⁸⁵

Hydrophobic tagging offers a different mean to achieve protein degradation. The approach *de facto* induces increased lipophilicity of the ligand, which implies that optimisation of the molecules towards drug-like properties needs to be tightly controlled.

3.3. Degradation by heterobifunctional degraders

The third and perhaps most significant type of protein degraders are heterobifunctional molecules, also known as PROTACs (proteolysis targeting chimeras) or SNIPERS (specific and non-genetic IAP-dependent protein erasers), which offer a rational approach for degrading a protein of interest (POI).^{69,86} These molecules combine a ligand to the POI and a ligand to an E3 ligase, connected by a linker of varying length. The formation of a ternary complex between the POI, E3 ligase and the bifunctional molecule leads to ubiquitination of the POI and ultimately to its degradation by the 26S proteasome. With this mechanism, the degrader acts as a transient molecular 'glue', which can repeat its action after ubiquitination or destruction of the POI and the molecule therefore acts as a catalyst. The approach can leverage small molecule binders to several E3 ligases, including mouse double minute 2 (MDM2),⁸⁷ cellular inhibitor of apoptosis protein 1 (cIAP1),^{88,89} von Hippel-Lindau (VHL),^{90–92} and cereblon (CRBN).^{93–96}

In principle, the ligands do not require functional activity for either protein. In fact, protein degradation is an opportunity to repurpose protein ligands which lack functional activity. For example, a heterobifunctional degrader of the bromodomain-containing transcriptional regulator TRIM24, dTRIM24 (Fig. 7), showed degradation-dependent enhanced anti-proliferation activity, while the potent bromo-domain inhibitor was devoid of activity.⁹⁷ In another example, GSK4027, a dual inhibitor of the bromodomains of two epigenetic proteins involved in immune functions, p300/CBP-associated factor (PCAF) and general control nonderepressible 5 (GCN5), displayed limited effect on the release of inflammatory cytokines in LPS-stimulated macrophages. Conversion to the thalidomide-based degrader GSK983 (Fig. 7) led to degradation of PCAF/GCN5 and potent modulation of cytokine release in macrophages and dendritic cells, mimicking genetic PCAF knock-down.⁹⁸

The applicable target space is being constantly expanded and includes kinases,^{99–103} epigenetic regulators,^{98,104–108} nuclear receptors,^{109–111} transcriptional regulators,^{97,112} tau protein,¹¹³ and ligases.^{114,115} So-called homo-PROTACs, dimerizing two E3 ligase binders, leading to self-directed ubiquitination and selective, proteasome-dependent degradation, has also been demonstrated for VHL,¹¹⁶ and CRBN.¹¹⁷ Degradation of transmembrane proteins has been shown for the receptor tyrosine kinases EGFR, HER2 and c-Met. Inhibi-

tion of cell proliferation was found to be more potent, and a more durable and sustained downstream signalling response with respect to a matched inhibitor, containing an inactive E3-ligase ligand, was observed, highlighting the advantages of this modality.¹¹⁸

A key step in this MOA is the formation of the ternary complex, as it determines efficacy and selectivity of the process.¹¹⁹ Complex formation is governed by attractive (positive cooperativity) or repulsive (negative cooperativity) interactions between the POI and the E3 ligase. At high degrader concentrations, binary complexes are formed, which prevents degradation,¹²⁰ and this so-called 'hook-effect' can render prediction of pharmacodynamic effects difficult. The relationship of complex formation and protein degradation has been demonstrated for a set of degraders, which target the bromodomain of BET proteins and VHL.¹²¹ In this study, a strong correlation between the half-life of the ternary complexes, as measured by surface plasma resonance, and the initial rate of degradation of the corresponding BET proteins was observed.

Despite the importance of ternary complex formation, optimisation is not trivial as cooperativity is not correlated with degradation efficiency. When studying MZ1 (Fig. 7), a degrader composed of the BRD4 ligand JQ1 and a VHL binder, the crystal structure of the ternary complex revealed a tight interface between the binding partners and highlighted the role of the degrader and its linker as a PPI stabiliser between the POI and the ligase, resulting in positive cooperativity.¹²² Intuitively, positive cooperativity should lead to more productive complex formation and better degradation. However, investigation of cereblon-BRD4 complexes revealed that effective degradation does not necessarily seem to require tight cooperative binding. In fact, efficient degradation was observed even with complexes having negative cooperativity.¹²³ PROTACs containing a covalent ligand binding to the POI would preclude the catalytic mechanism of the

Fig. 7 Representative examples of heterobifunctional degraders.

heterobifunctional degrader, but in principle could lead to stoichiometric degradation. This might be of interest for targets where only weak non-covalent binders are available. To investigate this further, covalent PROTACs targeting for Bruton's Tyrosine kinase (BTK) were constructed from the known BTK inhibitor ibrutinib, using IAP or CRBN as the ligase recruiting moiety. Surprisingly, no degradation of BTK using the covalent bifunctional molecule was observed, even at concentrations where full engagement of the POI and the ligase was demonstrated. However, the matched non-covalent molecule, devoid of the Michael acceptor, showed efficient degradation. The reasons for this somewhat surprising result are not completely understood but point to a critical role of catalysis for degradation.¹²⁴

Interestingly, heterobifunctional degraders provide the *a priori* unexpected opportunity to increase selectivity for a protein among closely related proteins. The PPIs which govern formation of the ternary degradation complex leverage differences outside the binding site of the POI ligand. This principle was first realised for BET proteins using the pan-inhibitor JQ1,¹²⁵ and has been extended to kinases.^{126,127}

Their MOA, larger size and floppy nature, renders bifunctional degrader drug discovery more complex than a conventional small molecule project, *a priori*. This is due to the challenges of developing the structure–activity relationship (SAR) in a larger chemical space and for a more complex mechanism of action, where efficacy and selectivity are not trivial to assess. In addition, these degraders are characterised by high molecular weight and, dependent on their recruiting ligands, potentially high lipophilicity, which can result in poor cell permeability, metabolic instability, low oral bioavailability and an increased risk of off-target effects. It is expected that a thorough analysis of their molecular properties will lead to a deeper understanding on the requirements for cell permeable, metabolically stable and orally available degraders. Recently, in the development of a degradation probe for the putative transcription regulator pirin, focus on properties such as linker length, log *D* and hydrogen-bond donor count led to a highly cell-active molecule from a barely active precursor.¹²⁸

Degradation selectivity, and efficacy are not yet fully understood, although impressive progress has been made, culminating with a degrader of the oestrogen receptor moving into phase I clinical studies.¹²⁹ The increasing number of methods to assess ternary complex formation including in cells,^{121,130} and to measure degradation over the whole proteome, the availability of X-ray structures, and progress in the simulation of ternary complexes^{127,131} is likely to further aid the design of heterobifunctional degraders. So far, the choice of E3-ligase recruiting moieties is limited but this is likely to be expanded in the near future.

Overall, the protein degradation MOA can be pursued through several strategies all aiming at directly or indirectly inducing degradation by the proteasomal machinery. Interestingly, other approaches are now being directed at exploiting other degradation mechanisms such as proteolysis in lysosomes through other drug modalities, as illustrated by

LYTAC,¹³² and chaperone mediated autophagy.¹³³ Very recently, a proof of concept study showed that extracellular POI's might also be amenable to targeted degradation by a heterobifunctional degrader. A recombinant eGFP-HT7 fusion protein, representing the extracellular POI, was linked to VUF11207, a known binder of the GPCR CXCR7, which causes receptor internalization upon binding. Investigating the MOA of these so-called endosome targeting chimeras (ENDTACs) revealed that the POI was internalized and degraded *via* the endosomal-lysosomal degradation pathway.¹³⁴

4. Downregulation of protein levels at the mRNA and ncRNA level

The principle of downregulating protein levels by intervention at the mRNA level has become well established with the development of oligonucleotides. Several entities have reached the market, both for antisense oligonucleotides (ASO) (fomivirsen, mipomersen, nusinersen, inotersen and eteplirsen) and siRNA (patisiran). These approaches offer the opportunity to systematically target a mRNA sequence leading to its degradation and therefore progressive reduction of protein levels, depending on protein turn over. However, major limitations hamper the broad applicability of these modalities, most notably their limited biodistribution. Indeed, oligonucleotides distribute preferentially to the liver, kidney and spleen, limiting the range of opportunities to modulate targets at the mRNA level in other tissues.

In this respect, other strategies taking advantage of this MOA are needed. Small molecules typically possess the ability to diffuse across all tissues, including the brain upon rigorous design. However, their ability to interact with RNA in a productive way has long been considered not possible due to the nature of RNA, made up of predominantly flat aromatic rings and a negatively charged backbone. With progress in structural biology, it is now known that RNA forms secondary, tertiary and quaternary structures, which provide binding pockets for small molecules. Furthermore RNA species are often associated with proteins and the formed interfaces represent additional opportunities for ligands to interact.^{135–137} The realisation of the potential to leverage small molecules to modulate RNA has led to a steep increase in research in the field, including the creation of several start-up companies.

However, targeting RNA at first intent is not obvious. Thus, many small molecules known to interact with RNA have been identified indirectly, typically during elucidation of the MOA after phenotypic screening. In the context of modulating RNA to downregulate a protein, one prominent example is the identification of small molecule modulators of proprotein convertase subtilisin/kexin type 9 (PCSK9) translation. PCSK9 controls the level of cholesterol by binding to the LDL receptor and promoting its degradation, which leads to increased plasma cholesterol levels, and is therefore associated with cardiovascular diseases.¹³⁸ Drug discovery efforts against this target have been successful in the form of antibodies with alirocumab and evolocumab being approved in recent years.

Due to the inconvenient route of administration of antibodies and the higher costs for this indication, many approaches have been directed at disrupting the interaction between PCSK9 and the LDL receptor through alternative modalities with the potential for oral delivery and reduced costs. The typical challenges of targeting a PPI has perhaps unsurprisingly led to many failures in these attempts. To circumvent these challenges, a team at Pfizer aimed to identify molecules capable of decreasing accumulation of PCSK9 by running a high throughput phenotypic screen in CHO cells.¹³⁸ Interestingly, a small molecule that reduced the cellular secretion of PCSK9 in a selective manner without affecting cell viability was identified (Fig. 8). While of somewhat modest activity ($IC_{50} = 4.8 \mu M$), the MOA was thoroughly studied. The compound was not cytotoxic, was not inducing PCSK9 degradation or acting *via* another pathway, as counter-screening against a panel of 113 targets showed. The observed changes in PCSK9 levels were rapid, suggesting that the compound intervened at the post-transcriptional stage. Indeed, mRNA levels of PCSK9 were unaffected. Consequently, the team focused their attention on protein synthesis and could eventually elucidate the MOA as being a reduction of PCSK9 synthesis at the translational level by binding to the 80S ribosome and stalling translation around codon 34. The compound was also evaluated *in vivo* and induced a reduction in systemic PCSK9 and cholesterol levels after oral administration.¹³⁹

The PCSK9 example demonstrates the possibility to downregulate a target by interacting with RNA species and proteins responsible for its translation. Although the MOA was a consequence of phenotypic screening rather than targeting this MOA in a rational manner, it illustrates how the interaction of RNA with small molecules may lead to a range of biological outcomes. In fact, other RNA species may represent novel targets for small molecules. For example, riboswitches, which are non-coding RNA elements in the 5'-UTR region of bacterial genes, that can recognise a small molecule and alter translation, are of high interest in the context of antibiotics. Scientists at Merck developed a novel screening strategy based on size-exclusion chromatography and affinity mass spectrometry. Using this strategy, small molecule libraries were rationally screened against five bacterial riboswitches.¹⁴⁰ For example, 53 000 compounds were tested against three constructs of the FMN riboswitch consisting of the wild type, a mutant and a scrambled version. The screen delivered 22 compounds selective for the wild type construct and 53 compounds selective for the mutant (see Fig. 8 for an example). A range of compounds were also shown to bind to the scrambled FMN RNA which could represent promiscuous RNA binders.

Beyond driving downregulation at the translational machinery level or targeting bacterial riboswitches, interacting with human non-coding RNA may also provide novel opportunities. Outside the sphere of protein-coding RNA, a breadth of RNA species appears to be increasingly associated with the modulation of biological processes. For example, the small

Fig. 8 Examples of small molecule RNA binders.

non-coding RNAs called microRNAs (miR) are involved in the regulation of mRNA and therefore protein translation, and targeting these will be discussed in section 5 in the context of upregulation. At the other end of the spectrum in terms of size, long non-coding RNA, usually consisting of over 200 nucleotides, may represent additional targets for the modulation of disease phenotypes. An example is the metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) long non-coding RNA, which is believed to play a role in cancer due to its increased expression in multiple cancers.¹⁴¹ MALAT1 forms tertiary structures including a triple helix, which has been targeted with small molecules. Starting from a known RNA binding scaffold, a series of binders was optimised, and binding resulted in a conformational change, essentially destabilising the triple helix (Fig. 8).¹⁴² In a separate effort, a small molecule microarray approach was used to identify MALAT1 binders.¹⁴³ The compounds were subsequently shown to decrease the level of MALAT1 in an organoid model of mammary tumours. Using a FRET assay, two different scaffolds were shown to bind MALAT1 with modest affinity (IC_{50} values = 1.3 and 2.5 μM). The switch of conformation from a triple helix is likely resulting in increased degradation of the RNA by RNases thus leading to decreased levels of the transcript.

Modulating mRNA levels may also be achieved by targeted degradation of RNA. Similarly, to the principle of protein degraders discussed in section 3, bifunctional molecules may be designed to bind to a mRNA of interest on one end, and on the other end to a degrading enzyme such as ribonucleases (RNases). The principle has been demonstrated on miR and will be discussed in section 5.3, but the same strategy should also be applicable to degrade mRNA and therefore downregulate proteins. Instead of recruiting a degrading enzyme, the use of a natural product causing DNA damage was recently applied to mRNA after a first application to miRs (section 5.3). In the context of the neuromuscular disease Myotonic Dystrophy 1, transcription of a CTG repeat in the dystrophin myotonia protein kinase (DMPK) gene results in CUG RNA repeats which form loops and sequester proteins involved in the regulation of pre-mRNA. Using a small molecule binder to the loop and dimerising it for avidity, a conjugate was generated upon coupling with Bleomycin A5.¹⁴⁴ The

resulting molecule, named Cugamycin, promoted cleavage of the repeat in patient-derived fibroblasts and rescued pre-mRNA splicing defects.

When compared to classical modalities targeting RNA such as ASO and siRNA, the prospect of targeting both coding and non-coding RNA species with small molecules enables MOAs capable of deeply modulating cellular processes while retaining cell permeability, oral administration and tissue distribution. However, major progress is still required to identify drug-like, tractable scaffolds. The structural determinants to bind RNA remain poorly characterised despite some emerging analyses.¹⁴⁵ The development of novel screening paradigms, such as the affinity MS strategy described above, should open the way to larger and more diverse screening campaigns and the identification of a broader set of RNA binders from which new structural elements could be derived.

5. Modulating protein expression, particularly enhancing expression, via RNA

As an alternative to protein inhibition or removal, upregulation of genes and proteins can be performed in a number of ways, from activating pathways *via* direct target modulation through to the direct administration of DNA or RNA or modulation of gene transcription and translation. Historically, pathway activation was achieved with specific agonists to targets such as GPCRs (*e.g.* beta-1 adrenergic receptor with small molecule agonists or glucagon-like peptide 1 receptor (GLP-1R) with peptide agonists), to nuclear hormone receptors such as retinoic acid receptor (RAR) or by enzyme activation with examples including glucokinase and AMP-activated protein kinase (AMPK). However, a variety of approaches are now emerging based on new modalities such as clustered regularly interspaced short palindromic repeats (CRISPR),^{146–148} or modified messenger RNA (mRNA).¹⁴⁹ The discovery of novel biology and therefore new potential MOAs are opening up novel possibilities including the stabilisation of PPIs (see section 2) and modulation of the activity of RNA. As access to genomic data increases and our ability to utilise this data to identify, validate and understand the genes responsible for disease deepens, additional opportunities to replace missing or dysfunctional proteins are arising. Gene therapy has often been hampered by the lack of delivery systems to enable effective and targeted delivery. Nevertheless, as this field advances rapidly, including for example delivery to specific organs or tissues, gene therapy will become more and more important to deliver a permanent introduction of a gene.^{150–152} However, in some cases a permanent introduction of a gene may not be desirable and in this case other approaches will be required.

Alternative technologies are now becoming available to provide new possibilities to enhance protein expression. For example, CRISPR gene editing is a rapidly developing technology which can also be used to activate gene transcription through

the recruitment of transcriptional activators.¹⁵³ However, the temporary upregulation of protein translation, avoiding insertional mutagenesis and giving a more controlled level of protein expression, allows careful control of activity depending on the requirements to treat a disease and also the ability to remove the protein if required. This section summarises some of the emerging approaches to deliver this through the modulation of RNA utilising a range of modalities (Fig. 9).

5.1. Modified messenger RNA

The discovery that *in vitro* transcribed modified mRNA encoding a protein of choice can be administered directly to utilise a cells' own protein synthesis factory to produce functional protein *in vivo* in a temporal and dose dependent manner has sparked a surge of research into this new modality.¹⁵⁴ Chemical modifications of mRNA including replacement of uridine-5'-triphosphate with pseudouridine-5'-triphosphate and cytidine with 5-methylcytidine during the *in vitro* transcription synthesis process provide mRNA with a reduced activation of the immune response.^{149,155} One of the first therapeutically aimed applications of this technology was the administration of mRNA encoding human vascular endothelial growth factor-A (VEGF-A) *via* an intramyocardial injection to give improved heart function after a myocardial infarction (MI).¹⁵⁶ The initial work was extended to show that a purified mRNA encoding VEGF-A administered in a biocompatible citrate-saline buffer could improve cardiac function in minipigs.¹⁵⁷ An intracardiac injection 7 days after induction of a MI led to improvements in a number of cardiac parameters including left ventricle ejection fraction, cardiac systolic function and contractility and also attenuated fibrosis. Modified mRNA is now being applied across different disease areas especially cancer immunotherapy either as a vaccine or as an encoder of immune response modulators such as cytokines.¹⁵⁴ mRNA is also being applied in alternative vaccine therapy for example against infectious diseases and allergy as well as diseases such as cystic fibrosis.¹⁵⁸ One of the biggest challenges with the administration of mRNA is specific delivery to tissues.¹⁵⁹ Still, progress is now being made in terms of increased understanding of mRNA cellular uptake and alternative targeted delivery strategies. For example, a nano-formulation consisting of hyperbranched poly(beta amino esters) to give stable polyplexes was suitable for inhalation and led to a localised delivery throughout the lung of a luciferase protein produced from luciferase mRNA.¹⁶⁰ Although this technology is still in development, a range of clinical trials are ongoing for the potential treatment of different diseases. As a recent example, the aforementioned mRNA encoding VEGF-A was injected intradermally into the forearm of men with type 2 diabetes mellitus leading to increased VEGF-A protein levels near the treated areas and enhanced basal skin blood flow.¹⁶¹

5.2. Activation of the promoter region with small activating RNA

Beyond the successful reduction of gene expression with oligonucleotides such as ASOs and siRNA,¹⁶² short double-

Fig. 9 Overview of strategies to increase protein translation.

stranded RNA oligonucleotides have also been shown to induce gene expression by targeting the promoter regions of genes to activate their transcription.¹⁶³ These small activating RNAs (saRNAs) have similarities to siRNA in that they require the Argonaute-2 protein for their mechanism of action, but they are distinct in their kinetics and ability to selectively induce expression of the target gene in the nucleus. For example, a saRNA has been designed to target the transcription factor CCATT/enhancer binding protein alpha (CEBPA) which encodes the protein CEBP/α.¹⁶⁴ CEBP/α has been shown to be downregulated in hepatocellular carcinoma (HCC) and is associated with poor survival. Hotspots for saRNAs targeting the CEBPA gene were identified through a bioinformatics analysis and subsequent synthesis of a series of oligonucleotides to walk across the nucleotides of the two hotspots. This revealed a sequence which upregulated the mRNA levels of CEBP/α 2.5-fold in an HCC cell line. The initial hit molecules were modified to introduce different arrangements of 2'-OMe base modifications to prevent stimulation of the immune system. Two of the modified methylation patterns were well tolerated to maintain the saRNA activity and one of these did not stimulate the immune system as measured by the secretion of TNFα and IFNα in PBMCs from two donors. The lead molecule, CEBPA-51 was subsequently taken into phase 1 clinical trials for patients with liver cancer.

5.3. Increasing mRNA half-life by preventing micro RNA-based degradation

Gene expression is regulated at the mRNA level by interaction with non-coding RNAs such as miRs and dysregulation of miRs is a common feature of several diseases.¹⁶⁵ miRs are short, around 22 nucleotides in length, single-stranded RNAs that target complementary oligonucleotide sequences usually located at the 3'-untranslated region in mRNAs. These interactions inhibit mRNA translation or cause mRNA degradation, and therefore suppress protein expression. Many mRNAs can be targeted by a single miR and therefore manipulating miR expression or function can cause a significant change. The function of miRs is still not well understood and a lot of research is ongoing to build understanding of their function and mechanisms which will provide more opportunities to effectively target them in disease. Hence, when miRs are inhibited there is a greater availability of mRNA for translation which can lead to an increase in protein expression.

The most commonly used approach to inhibit miRs is by using anti-miR ASOs.¹⁶⁶ ASOs usually consist of a single strand of 16–20 stabilised nucleotides which binds a target RNA sequence through Watson–Crick base pairing and a number of these molecules are undergoing clinical development.

As referred to in section 4, approaches to target RNA using small molecules are also being developed.¹⁶⁷ This strategy

has also been shown to target miRs. In a recent example, a bioinformatics approach to identify potentially druggable RNA targets, named Inforna, was applied to the discovery of a small molecule inhibiting the production of mature miR-210.¹⁶⁸ miR-210 is involved in the repression of glycerol-3-phosphate dehydrogenase 1-like enzyme (GPD1L) translation. Following binding to the dicer site of the miR precursor, the small molecule, named Targapremir-210, derepresses GDPL1 which subsequently suppresses prolyl hydroxylase activity and reduces HIF-1 α levels, eventually leading to apoptosis of triple negative breast cancer cells under hypoxic conditions. Targapremir-210 was also efficacious *in vivo* as shown in a mouse xenograft model of hypoxic negative breast cancer. A major challenge presented by targeting RNA with small molecules is specificity. With this prospect in mind, the specificity and selectivity of targapremir-210 against other RNAs was explored. The compound did not bind to RNA where the Dicer site was removed and in a cellular study against a set of 28 miRs associated with hypoxia the compound only affected the levels of mature miR-210. In addition, testing the compound against 2500 miRs revealed that targapremir-210 had a similar specificity profile to a miR-210 antagomir. Direct target engagement was shown using a method known as chemical cross-linking and isolation by pull-down (Chem-CLIP) in which the lead molecule, Targapremir-210 in this case, was derivatised with a chloroambucil cross-linking motif and a biotin moiety for subsequent pull down experiments with streptavidin.¹⁶⁹ The Chem-CLIP experiment in the MDA-MB-231 triple negative breast cancer cells grown under hypoxic conditions showed direct target engagement of the compound with the hairpin precursor of miR-210. This example highlights the emerging understanding and utility of small molecules to target specific RNA motifs leading to disease relevant phenotypes.

As alluded to in section 4, promoting the degradation of RNA species with bivalent molecules has recently been proposed as a strategy to downregulate miR levels (Fig. 10). One approach consists of tethering an RNA binder to an RNase recruiter. For the first chimera, named ribonuclease targeting chimera (RIBOTAC), a 2'-5' polyA oligonucleotide was conjugated to a small molecule binder of miR96.¹⁷⁰ In this construct,

the oligonucleotide drives the dimerisation and subsequent activation of RNase L. The RIBOTAC resulted in degradation of both pri-miR96 and mature miR96 in a range of cancer cell lines. Degradation of the miR led to stabilisation of the FOXO-1 mRNA leading to increased protein levels, and a pro-apoptotic phenotype. Interestingly, the RIBOTAC resulted in the same level of apoptosis as the miR96 binder alone albeit at a 2.5-fold lower concentration, highlighting the catalytic nature of the mechanism. Similarly to the example targeting CUG repeats and discussed in section 4, another approach to degrade miR with bivalent molecules is the conjugation of a RNA binder to a DNA damage promoter.¹⁷¹ Conjugation of the miR96 small molecule binder described above to bleomycin A5, which activates oxygen resulting in nucleotide cleavage, led to a 100-fold increase in the affinity of the natural product for the miR compared to DNA and resulted in the cleavage of miR96 and a similar FOXO-1 and apoptotic outcome as described for the RIBOTAC.

5.4. Correction of gene function by targeting RNA splicing

A number of diseases are caused by the incorrect processing and splicing of RNA. The mechanism to process pre-mRNA into a mature mRNA is known as splicing and the U1-U2 major spliceosome is a critical component which acts to catalytically ligate exons and release an intron. This is a tightly controlled process regulated by a broad range of cofactors.¹⁷² A high percentage of disease-causing mutations, which lead to a range of genetic disorders, are caused by incorrect splicing. Hence, selectively modulating exon splicing can be a beneficial approach to positively impact genetic disorders.

One exciting approach has recently been reported for the treatment of spinal muscular atrophy (SMA), a rare autosomal recessive neuromuscular disease, caused by a defect in the survival motor neuron (SMN) protein.^{173,174} In SMA, the SMN1 gene is indeed deleted or has a loss-of-function mutation leading to degeneration of motor neurons. In humans, a second related gene exists, SMN2, but altered splicing and exclusion of exon 7, leads to only 10–20% production of full length SMN mRNA and protein, and the remaining SMN

Fig. 10 Principles of RNA degraders.

Fig. 11 Small molecules splicing modulators.

mRNA encodes an unstable protein. Hence a strategy to restore levels of functional SMN protein and treat SMA is to modulate the splicing of SMN2 pre-mRNA to produce full length SMN mRNA. While nusinersen, a splice correcting ASO, was approved by the FDA in December 2016,¹⁷⁵ small molecule efforts have also been directed at correcting splicing. Thus, a phenotypic screen using a motor neuron cell line expressing SMN2 reporters led to the discovery of compounds enhancing inclusion of exon 7, *i.e.* the desired activity on splicing, which subsequently increased SMN levels.¹⁷³ The mechanism of these compounds was elucidated using a variety of methods, such as RNAseq, chimera and mutation studies, NMR experiments in the presence of double strand (ds) RNA and computational modelling, and was shown to consist in stabilisation of the dsRNA structure formed between the pre-mRNA of SMN2 and the U1 snRNP complex, which is a key part of the spliceosome. The hit molecule identified in the phenotypic screen was optimised through multi-parameter optimisation to give the lead compound known as branaplam (Fig. 11), which is now undergoing human clinical trials for SMA.¹⁷⁶ Different classes of compounds which promote the inclusion of exon 7 in the SMN2 mRNA have also been discovered and optimisation of these early hits has led to the discovery of risdiplam (Fig. 11), which has also reached the clinic.¹⁷⁷

These examples highlight that it is possible to discover safe and efficacious modulators of RNA splicing and provide a new class of small molecule therapeutics. This approach has the potential to treat a range of diseases caused by dysfunctional RNA splicing.

5.5. Targeting RNA modifying proteins

Another emerging area of biology leveraged to upregulate protein levels relates to enzyme-catalysed covalent modifications of RNA, which impact RNA structure, stability and function. These modifications can affect the translation of RNA to proteins as well as nuclear export, degradation and splicing.¹⁷⁸ These mechanisms, called epitranscriptomics, involve proteins known as RNA-modifying proteins (RMPs), a class of enzymes responsible for modifying RNA through covalent modification, or recognising the modified RNA structure and removing covalent modifications.¹⁷⁹ Many of the RMPs responsible for these modifications are unknown or not yet fully characterised, but examples include S-adenosyl-L-methionine dependent methyltransferases which methylate purine or pyrimidine bases, RNA demethylases belonging to the ALKB family of enzymes, and RMPs which isomerise the

Fig. 12 Examples of RNA nucleoside modifications.

nucleosides of RNA, including pseudouridine synthase (Fig. 12). Whilst this field is still in its infancy, initial data suggest that some epitranscriptomic mechanisms are altered in some human diseases including cancer.¹⁸⁰

One of the most investigated mRNA modifications involves 6-methyladenosine (m6A).¹⁸¹ Analytical methods have been developed to study this modification and the enzymes which methylate and demethylate this position have been identified as methyltransferase-like proteins 3 and 14 (METTL3 and METTL14) for methylation, and FTO and ALKBH5 for demethylation respectively. Interestingly, depletion of METTL3 using short hairpin RNA promotes differentiation of human hematopoietic stem/progenitor cells leading to a reduction in cell proliferation and to a delay in leukemia progression in mice transplanted with the leukaemia MOLM-13 cell line.¹⁸²

To date very few RMP inhibitors are known. However, due to their similarities with chromatin modifying enzymes it is reasonable to speculate that these RMPs will indeed be druggable. In addition, structural information is available for a range of RMPs, such as RNA methyltransferases, which can be utilised to provide hypotheses and enable structure based drug design.¹⁸³

Opportunities and approaches to specifically enhance protein expression *via* RNA are now emerging with compounds progressing into and through clinical trials. Some strategies are still very early and will undoubtedly have challenges ahead. However, some of these challenges, for example delivery of modified mRNA or rational design approaches for RNA binding or RNA splicing modulation are a focus of many researchers and solutions will emerge, allowing additional possibilities in this field. Enhancing protein expression carries great potential to impact a range of diseases including those where a gene is missing, or mutated, or where a boost of a protein is required to help the human body tackle an ailment and therefore these emerging MOAs will see greater attention in drug discovery in the coming years.

6. Conclusion

The emerging MOAs described in this review illustrate the expanding arsenal of strategies to prosecute biological targets. Many more strategies are developing, especially molecules with multivalency. For example, a class of bivalent molecules called antibody-recruiting small molecules are being leveraged in

immuno-oncology and infectious diseases.¹⁸⁴ Multimeric ligands can also enable receptor activation as demonstrated with bivalent peptides inducing Met receptor dimerisation and activation of the corresponding signalling pathway.¹⁸⁵

As our understanding of human biology and the function of biological systems increases as well as the development of new, creative technologies expand, the opportunities for therapeutic intervention will be enhanced. Whilst many drug discovery efforts are focused on established modes of action, and will continue to be so, new possibilities are emerging. Many targets, previously deemed undruggable, are now being challenged with new strategies with scientists armed with a more diverse toolbox to find ways to modulate these targets. What was undruggable before is perhaps better described as that we had not found a way to drug before.

Many of these new approaches, including those described herein, are relatively novel from a drug development perspective with biology and hit molecules either only just being discovered, through to approaches, where molecules have entered and are now progressing through clinical trials. While some of these possibilities originated from understanding the MOA of molecules discovered with the sought after phenotype from phenotypic screens, a clearer understanding driven by biological and chemical biology approaches is now developing to provide the knowledge and tools to target these MOAs in a more rational manner.¹⁸⁶ The curiosity and creativity of scientists to learn from naturally occurring biological mechanisms and understand the key drivers in human disease are driving the application of these methods from a drug discovery viewpoint. As with all new technologies, these new MOAs will face further challenges as they move forward and problem solving will be required on many fronts to successfully deliver new medicines. Some will hopefully make it through to become medicines of the future, some may not. However, patients clearly need new, differentiated medicines which can truly impact their lives, and driving molecules with these new modes of action forward to increase the arsenal in which scientists can approach and cure disease is an exciting prospect.

Conflicts of interest

There are no conflicts to declare.

Notes and references

- 1 E. Valeur and P. Jimonet, *J. Med. Chem.*, 2018, **61**, 9004–9029.
- 2 J. A. Doudna and E. Charpentier, *Science*, 2014, **346**, 1258096.
- 3 C. Fellmann, B. G. Gowen, P.-C. Lin, J. A. Doudna and J. E. Corn, *Nat. Rev. Drug Discovery*, 2017, **16**, 89–100.
- 4 V. K. Sharma, P. Rungta and A. K. Prasad, *RSC Adv.*, 2014, **4**, 16618–16631.
- 5 A. C. Lai and C. M. Crews, *Nat. Rev. Drug Discovery*, 2017, **16**, 101–114.
- 6 E. Valeur, S. M. Guéret, H. Adihou, R. Gopalakrishnan, M. Lemurell, H. Waldmann, T. N. Grossmann and A. T. Plowright, *Angew. Chem., Int. Ed.*, 2017, **56**, 10294–10323.
- 7 D. Bier, P. Thiel, J. Briels and C. Ottmann, *Prog. Biophys. Mol. Biol.*, 2015, **119**, 10–19.
- 8 S. A. Andrei, E. Sijbesma, M. Hann, J. Davis, G. O'Mahony, M. W. D. Perry, A. Karawajczyk, J. Eickhoff, L. Brunsvel, R. G. Doveston, L.-G. Milroy and C. Ottmann, *Expert Opin. Drug Discovery*, 2017, **12**, 925–940.
- 9 P. Thiel, M. Kaiser and C. Ottmann, *Angew. Chem., Int. Ed.*, 2012, **51**, 2012–2018.
- 10 J. Liu, J. D. Farmer, W. S. Lane, J. Friedman, I. Weissman and S. L. Schreiber, *Cell*, 1991, **66**, 807–815.
- 11 E. J. Brown, M. W. Albers, T. B. Shin, K. Ichikawa, C. T. Keith, W. S. Lane and S. L. Schreiber, *Nature*, 1994, **369**, 756–758.
- 12 A. Peyroche, B. Antonny, S. Robineau, J. Acker, J. Cherfils and C. L. Jackson, *Mol. Cell*, 1999, **3**, 275–285.
- 13 J. J. Tesmer, R. K. Sunahara, A. G. Gilman and S. R. Sprang, *Science*, 1997, **278**, 1907–1916.
- 14 X. Tan, L. I. A. Calderon-Villalobos, M. Sharon, C. Zheng, C. V. Robinson, M. Estelle and N. Zheng, *Nature*, 2007, **446**, 640–645.
- 15 L. B. Sheard, X. Tan, H. Mao, J. Withers, G. Ben-Nissan, T. R. Hinds, Y. Kobayashi, F.-F. Hsu, M. Sharon, J. Browse, S. Y. He, J. Rizo, G. A. Howe and N. Zheng, *Nature*, 2010, **468**, 400–405.
- 16 W. Rehman, L. M. Arfons and H. M. Lazarus, *Ther. Adv. Hematol.*, 2011, **2**, 291–308.
- 17 C. Galustian and A. Dagleish, *Expert Opin. Pharmacother.*, 2009, **10**, 125–133.
- 18 G. Petzold, E. S. Fischer and N. H. Thomä, *Nature*, 2016, **532**, 127–130.
- 19 S. J. Hughes and A. Ciulli, *Essays Biochem.*, 2017, **61**, 505–516.
- 20 F. Chiti and C. M. Dobson, *Annu. Rev. Biochem.*, 2006, **75**, 333–366.
- 21 B. Ng, L. H. Connors, R. Davidoff, M. Skinner and R. H. Falk, *Arch. Intern. Med.*, 2005, **165**, 1425–1429.
- 22 P. Hammarström, R. L. Wiseman, E. T. Powers and J. W. Kelly, *Science*, 2003, **299**, 713–716.
- 23 G. J. Miroy, Z. Lai, H. A. Lashuel, S. A. Peterson, C. Strang and J. W. Kelly, *Proc. Natl. Acad. Sci. U. S. A.*, 1996, **93**, 15051–15056.
- 24 S. L. Adamski-Werner, S. K. Palaninathan, J. C. Sacchettini and J. W. Kelly, *J. Med. Chem.*, 2004, **47**, 355–374.
- 25 M. S. Maurer, J. H. Schwartz, B. Gundapaneni, P. M. Elliott, G. Merlini, M. Waddington-Cruz, A. V. Kristen, M. Grogan, R. Witteles, T. Dany, B. M. Drachman, S. J. Shah, M. Hanna, D. P. Judge, A. I. Barsdorf, P. Huber, T. A. Patterson, S. Riley, J. Schumacher, M. Stewart, M. B. Sultan, C. Rapezzi and ATTR-ACT Study Investigators, *N. Engl. J. Med.*, 2018, **379**, 1007–1016.
- 26 C. E. Bulawa, S. Connelly, M. Devit, L. Wang, C. Weigel, J. A. Fleming, J. Packman, E. T. Powers, R. L. Wiseman,

- T. R. Foss, I. A. Wilson, J. W. Kelly and R. Labaudinière, *Proc. Natl. Acad. Sci. U. S. A.*, 2012, **109**, 9629–9634.
- 27 R. Sant'Anna, P. Gallego, L. Z. Robinson, A. Pereira-Henriques, N. Ferreira, F. Pinheiro, S. Esperante, I. Pallares, O. Huertas, M. R. Almeida, N. Reixach, R. Insa, A. Velazquez-Campoy, D. Reverter, N. Reig and S. Ventura, *Nat. Commun.*, 2016, **7**, 10787.
- 28 S. Classen, S. Olland and J. M. Berger, *Proc. Natl. Acad. Sci. U. S. A.*, 2003, **100**, 10629–10634.
- 29 E. Karakas, N. Simorowski and H. Furukawa, *Nature*, 2011, **475**, 249–253.
- 30 M. Tinti, F. Madeira, G. Murugesan, G. Hoxhaj, R. Toth and C. Mackintosh, *Database*, 2014, **2014**, bat085.
- 31 F. Madeira, M. Tinti, G. Murugesan, E. Berrett, M. Stafford, R. Toth, C. Cole, C. MacKintosh and G. J. Barton, *Bioinformatics*, 2015, **31**, 2276–2283.
- 32 M. Saha, A. Carriere, M. Cheerathodi, X. Zhang, G. Lavoie, J. Rush, P. P. Roux and B. A. Ballif, *Biochem. J.*, 2012, **447**, 159–166.
- 33 A. Ballone, F. Centorrino, M. Wolter and C. Ottmann, *J. Struct. Biol.*, 2018, **202**, 210–215.
- 34 L. R. Jagemann, L. G. Pérez-Rivas, E. J. Ruiz, J. A. Ranea, F. Sánchez-Jiménez, A. R. Nebreda, E. Alba and J. Lozano, *J. Biol. Chem.*, 2008, **283**, 17450–17462.
- 35 A. Fischer, A. Baljuls, J. Reinders, E. Nekhoroshkova, C. Sibilski, R. Metz, S. Albert, K. Rajalingam, M. Hekman and U. R. Rapp, *J. Biol. Chem.*, 2009, **284**, 3183–3194.
- 36 S. Ory, M. Zhou, T. P. Conrads, T. D. Veenstra and D. K. Morrison, *Curr. Biol.*, 2003, **13**, 1356–1364.
- 37 C.-H. Shen, P. Yuan, R. Perez-Lorenzo, Y. Zhang, S. X. Lee, Y. Ou, J. M. Asara, L. C. Cantley and B. Zheng, *Mol. Cell*, 2013, **52**, 161–172.
- 38 N. Dumaz and R. Marais, *J. Biol. Chem.*, 2003, **278**, 29819–29823.
- 39 M. Kilisch, O. Lytovchenko, E. C. Arakel, D. Bertinetti and B. Schwappach, *J. Cell Sci.*, 2016, **129**, 831–842.
- 40 L. M. Stevers, C. V. Lam, S. F. R. Leysen, F. A. Meijer, D. S. van Scheppingen, R. M. J. M. de Vries, G. W. Carlile, L. G. Milroy, D. Y. Thomas, L. Brunsveld and C. Ottmann, *Proc. Natl. Acad. Sci. U. S. A.*, 2016, **113**, E1152–1161.
- 41 K. Muda, D. Bertinetti, F. Gesellchen, J. S. Hermann, F. von Zweydford, A. Geerlof, A. Jacob, M. Ueffing, C. J. Gloeckner and F. W. Herberg, *Proc. Natl. Acad. Sci. U. S. A.*, 2014, **111**, E34–43.
- 42 L. Phan, P.-C. Chou, G. Velazquez-Torres, I. Samudio, K. Parreno, Y. Huang, C. Tseng, T. Vu, C. Gully, C.-H. Su, E. Wang, J. Chen, H.-H. Choi, E. Fuentes-Mattei, J.-H. Shin, C. Shiang, B. Grabiner, M. Blonska, S. Skerl, Y. Shao, D. Cody, J. Delacerda, C. Kingsley, D. Webb, C. Carlock, Z. Zhou, Y.-C. Hsieh, J. Lee, A. Elliott, M. Ramirez, J. Bankson, J. Hazle, Y. Wang, L. Li, S. Weng, N. Rizk, Y. Y. Wen, X. Lin, H. Wang, H. Wang, A. Zhang, X. Xia, Y. Wu, M. Habra, W. Yang, L. Pusztai, S.-C. Yeung and M.-H. Lee, *Nat. Commun.*, 2015, **6**, 7530.
- 43 H. Y. Qureshi, T. Li, R. MacDonald, C. M. Cho, N. Leclerc and H. K. Paudel, *Biochemistry*, 2013, **52**, 6445–6455.
- 44 N. Plotegher, D. Kumar, I. Tessari, M. Bruciale, F. Munari, L. Tosatto, E. Belluzzi, E. Greggio, M. Bisaglia, S. Capaldi, D. Aioanei, S. Mammi, H. L. Monaco, B. Samo and L. Bubacco, *Hum. Mol. Genet.*, 2014, **23**, 5615–5629.
- 45 Z. Xu, K. Graham, M. Foote, F. Liang, R. Rizkallah, M. Hurt, Y. Wang, Y. Wu and Y. Zhou, *J. Cell Sci.*, 2013, **126**, 4173–4186.
- 46 P. Jafar-Nejad, C. S. Ward, R. Richman, H. T. Orr and H. Y. Zoghbi, *Proc. Natl. Acad. Sci. U. S. A.*, 2011, **108**, 2142–2147.
- 47 L. M. Stevers, E. Sijbesma, M. Botta, C. MacKintosh, T. Obsil, I. Landrieu, Y. Cau, A. J. Wilson, A. Karawajczyk, J. Eickhoff, J. Davis, M. Hann, G. O'Mahony, R. G. Doveston, L. Brunsveld and C. Ottmann, *J. Med. Chem.*, 2018, **61**, 3755–3778.
- 48 A. H. de Boer and I. J. de Vries-van Leeuwen, *Trends Plant Sci.*, 2012, **17**, 360–368.
- 49 L. Camoni, S. Visconti and P. Aducci, *IUBMB Life*, 2013, **65**, 513–517.
- 50 I. J. De Vries-van Leeuwen, D. da Costa Pereira, K. D. Flach, S. R. Piersma, C. Haase, D. Bier, Z. Yalcin, R. Michalides, K. A. Feenstra, C. R. Jiménez, T. F. A. de Greef, L. Brunsveld, C. Ottmann, W. Zwart and A. H. de Boer, *Proc. Natl. Acad. Sci. U. S. A.*, 2013, **110**, 8894–8899.
- 51 R. G. Doveston, A. Kuusk, S. A. Andrei, S. Leysen, Q. Cao, M. P. Castaldi, A. Hendricks, L. Brunsveld, H. Chen, H. Boyd and C. Ottmann, *FEBS Lett.*, 2017, **591**, 2449–2457.
- 52 A. Kaplan, B. Morquette, A. Kroner, S. Leong, C. Madwar, R. Sanz, S. L. Banerjee, J. Antel, N. Bisson, S. David and A. E. Fournier, *Neuron*, 2017, **93**, 1082–1093.e5.
- 53 A. Kaplan, C. Ottmann and A. E. Fournier, *Pharmacol. Res.*, 2017, **125**, 114–121.
- 54 M. Molzan, S. Kasper, L. Röglin, M. Skwarczynska, T. Sassa, T. Inoue, F. Breitenbuecher, J. Ohkanda, N. Kato, M. Schuler and C. Ottmann, *ACS Chem. Biol.*, 2013, **8**, 1869–1875.
- 55 D. Bier, M. Bartel, K. Sies, S. Halbach, Y. Higuchi, Y. Haranosono, T. Brummer, N. Kato and C. Ottmann, *ChemMedChem*, 2016, **11**, 911–918.
- 56 C. Anders, Y. Higuchi, K. Koschinsky, M. Bartel, B. Schumacher, P. Thiel, H. Nitta, R. Preisig-Müller, G. Schlichthörl, V. Renigunta, J. Ohkanda, J. Daut, N. Kato and C. Ottmann, *Chem. Biol.*, 2013, **20**, 583–593.
- 57 S. A. Andrei, P. de Vink, E. Sijbesma, L. Han, L. Brunsveld, N. Kato, C. Ottmann and Y. Higuchi, *Angew. Chem., Int. Ed.*, 2018, **57**, 13470–13474.
- 58 E. Sijbesma, L. Skora, S. Leysen, L. Brunsveld, U. Koch, P. Nussbaumer, W. Jahnke and C. Ottmann, *Biochemistry*, 2017, **56**, 3972–3982.
- 59 K. K. Hallenbeck, J. L. Davies, C. Merron, P. Ogden, E. Sijbesma, C. Ottmann, A. R. Renslo, C. Wilson and M. R. Arkin, *SLAS Discovery*, 2018, **23**, 183–192.
- 60 E. Sijbesma, K. K. Hallenbeck, S. Leysen, P. J. de Vink, L. Skóra, W. Jahnke, L. Brunsveld, M. R. Arkin and C. Ottmann, *J. Am. Chem. Soc.*, 2019, **141**, 3524–3531.
- 61 D. Bier, R. Rose, K. Bravo-Rodriguez, M. Bartel, J. M. Ramirez-Angueta, S. Dutt, C. Wilch, F.-G. Klärner, E.

- Sanchez-Garcia, T. Schrader and C. Ottmann, *Nat. Chem.*, 2013, **5**, 234–239.
- 62 D. Bier, S. Mittal, K. Bravo-Rodriguez, A. Sowislok, X. Guillory, J. Briels, C. Heid, M. Bartel, B. Wettig, L. Brunsveld, E. Sanchez-Garcia, T. Schrader and C. Ottmann, *J. Am. Chem. Soc.*, 2017, **139**, 16256–16263.
- 63 M. Gao and J. Skolnick, *Proc. Natl. Acad. Sci. U. S. A.*, 2012, **109**, 3784–3789.
- 64 N. B. Struntz, A. Chen, A. Deutzmann, R. M. Wilson, E. Stefan, H. L. Evans, M. A. Ramirez, T. Liang, F. Caballero, M. H. E. Wildschut, D. V. Neel, D. B. Freeman, M. S. Pop, M. McConkey, S. Muller, B. H. Curtin, H. Tseng, K. R. Frombach, V. L. Butty, S. S. Levine, C. Feau, S. Elmiligy, J. A. Hong, T. A. Lewis, A. Vetere, P. A. Clemons, S. E. Malstrom, B. L. Ebert, C. Y. Lin, D. W. Felsher and A. N. Koehler, *Cell Chem. Biol.*, 2019, **26**, 711–723.
- 65 N. Mainolfi and T. Rasmusson, in *Annual Reports in Medicinal Chemistry*, ed. R. A. Goodnow, Academic Press, 2017, vol. 50, pp. 301–334.
- 66 Y. Zhang, C. Loh, J. Chen and N. Mainolfi, *Drug Discovery Today: Technol.*, 2019, **31**, 53–60.
- 67 C. M. Crews, G. Georg and S. Wang, *J. Med. Chem.*, 2016, **59**, 5129–5130.
- 68 C. M. Olson, B. Jiang, M. A. Erb, Y. Liang, Z. M. Doctor, Z. Zhang, T. Zhang, N. Kwiatkowski, M. Boukhali, J. L. Green, W. Haas, T. Nomanbhoy, E. S. Fischer, R. A. Young, J. E. Bradner, G. E. Winter and N. S. Gray, *Nat. Chem. Biol.*, 2018, **14**, 163–170.
- 69 I. Churcher, *J. Med. Chem.*, 2018, **61**, 444–452.
- 70 X. Long and K. P. Nephew, *J. Biol. Chem.*, 2006, **281**, 9607–9615.
- 71 C. De Savi, R. H. Bradbury, A. A. Rabow, R. A. Norman, C. de Almeida, D. M. Andrews, P. Ballard, D. Buttar, R. J. Callis, G. S. Currie, J. O. Curwen, C. D. Davies, C. S. Donald, L. J. L. Feron, H. Gingell, S. C. Glossop, B. R. Hayter, S. Hussain, G. Karoutchi, S. G. Lamont, P. MacFaul, T. A. Moss, S. E. Pearson, M. Tonge, G. E. Walker, H. M. Weir and Z. Wilson, *J. Med. Chem.*, 2015, **58**, 8128–8140.
- 72 S. W. Fanning, L. Hodges-Gallagher, D. C. Myles, R. Sun, C. E. Fowler, I. N. Plant, B. D. Green, C. L. Harmon, G. L. Greene and P. J. Kushner, *Nat. Commun.*, 2018, **9**, 2368.
- 73 L. H. Jones, *Cell Chem. Biol.*, 2018, **25**, 30–35.
- 74 A. Citri, I. Alroy, S. Lavi, C. Rubin, W. Xu, N. Grammatikakis, C. Patterson, L. Neckers, D. W. Fry and Y. Yarden, *EMBO J.*, 2002, **21**, 2407–2417.
- 75 N. Kerres, S. Steurer, S. Schlager, G. Bader, H. Berger, M. Caligiuri, C. Dank, J. R. Engen, P. Ettmayer, B. Fischerauer, G. Flotzinger, D. Gerlach, T. Gerstberger, T. Gmaschitz, P. Greb, B. Han, E. Heyes, R. E. Jacob, D. Kessler, H. Kölle, L. Lamarre, D. R. Lancia, S. Lucas, M. Mayer, K. Mayr, N. Mischerikow, K. Mück, C. Peinsipp, O. Petermann, U. Reiser, D. Rudolph, K. Rumpel, C. Salomon, D. Scharn, R. Schnitzer, A. Schrenk, N. Schweifer, D. Thompson, E. Traxler, R. Varecka, T. Voss, A. Weiss-Puxbaum, S. Winkler, X. Zheng, A. Zoephel, N. Kraut, D. McConnell, M. Pearson and M. Koegl, *Cell Rep.*, 2017, **20**, 2860–2875.
- 76 A. S. Waller, R. M. Sharrard, P. Berthon and N. J. Maitland, *J. Mol. Endocrinol.*, 2000, **24**, 339–351.
- 77 K. Peltonen, L. Colis, H. Liu, R. Trivedi, M. S. Moubarek, H. M. Moore, B. Bai, M. A. Rudek, C. J. Bieberich and M. Laiho, *Cancer Cell*, 2014, **25**, 77–90.
- 78 K. R. Simonetta, J. Taygerly, K. Boyle, S. E. Basham, C. Padovani, Y. Lou, T. J. Cummins, S. L. Yung, S. K. von Soly, F. Kayser, J. Kuriyan, M. Rape, M. Cardozo, M. A. Gallop, N. F. Bence, P. A. Barsanti and A. Saha, *Nat. Commun.*, 2019, **10**, 1402.
- 79 T. K. Neklesa and C. M. Crews, *Nature*, 2012, **487**, 308–309.
- 80 T. K. Neklesa, H. S. Tae, A. R. Schneekloth, M. J. Stulberg, T. W. Corson, T. B. Sundberg, K. Raina, S. A. Holley and C. M. Crews, *Nat. Chem. Biol.*, 2011, **7**, 538–543.
- 81 T. Xie, S. M. Lim, K. D. Westover, M. E. Dodge, D. Ercan, S. B. Ficarro, D. Udayakumar, D. Gurbani, H. S. Tae, S. M. Riddle, T. Sim, J. A. Marto, P. A. Jänne, C. M. Crews and N. S. Gray, *Nat. Chem. Biol.*, 2014, **10**, 1006–1012.
- 82 J. L. Gustafson, T. K. Neklesa, C. S. Cox, A. G. Roth, D. L. Buckley, H. S. Tae, T. B. Sundberg, D. B. Stagg, J. Hines, D. P. McDonnell, J. D. Norris and C. M. Crews, *Angew. Chem., Int. Ed.*, 2015, **54**, 9659–9662.
- 83 N. Gao, Y.-P. Huang, T.-T. Chu, Q.-Q. Li, B. Zhou, Y.-X. Chen, Y.-F. Zhao and Y.-M. Li, *Bioorg. Chem.*, 2019, **84**, 254–259.
- 84 N. Gao, T.-T. Chu, Q.-Q. Li, Y.-J. Lim, T. Qiu, M.-R. Ma, Z.-W. Hu, X.-F. Yang, Y.-X. Chen, Y.-F. Zhao and Y.-M. Li, *RSC Adv.*, 2017, **7**, 40362–40366.
- 85 Y. Shi, M. J. C. Long, M. M. Rosenberg, S. Li, A. Kobjack, P. Lessans, R. T. Coffey and L. Hedstrom, *ACS Chem. Biol.*, 2016, **11**, 3328–3337.
- 86 P. Ottis and C. M. Crews, *ACS Chem. Biol.*, 2017, **12**, 892–898.
- 87 A. R. Schneekloth, M. Pucheault, H. S. Tae and C. M. Crews, *Bioorg. Med. Chem. Lett.*, 2008, **18**, 5904–5908.
- 88 K. Sekine, K. Takubo, R. Kikuchi, M. Nishimoto, M. Kitagawa, F. Abe, K. Nishikawa, T. Tsuruo and M. Naito, *J. Biol. Chem.*, 2008, **283**, 8961–8968.
- 89 Y. Itoh, M. Ishikawa, M. Naito and Y. Hashimoto, *J. Am. Chem. Soc.*, 2010, **132**, 5820–5826.
- 90 P. Soares, M. S. Gadd, J. Frost, C. Galdeano, L. Ellis, O. Epemolu, S. Rocha, K. D. Read and A. Ciulli, *J. Med. Chem.*, 2018, **61**, 599–618.
- 91 A. Testa, X. Lucas, G. V. Castro, K.-H. Chan, J. E. Wright, A. C. Runcie, M. S. Gadd, W. T. A. Harrison, E.-J. Ko, D. Fletcher and A. Ciulli, *J. Am. Chem. Soc.*, 2018, **140**, 9299–9313.
- 92 D. L. Buckley, J. L. Gustafson, I. Van Molle, A. G. Roth, H. S. Tae, P. C. Gareiss, W. L. Jorgensen, A. Ciulli and C. M. Crews, *Angew. Chem., Int. Ed.*, 2012, **51**, 11463–11467.
- 93 J. Krönke, N. D. Udeshi, A. Narla, P. Grauman, S. N. Hurst, M. McConkey, T. Svinkina, D. Heckl, E. Comer, X. Li, C. Ciarlo, E. Hartman, N. Munshi, M. Schenone, S. L. Schreiber, S. A. Carr and B. L. Ebert, *Science*, 2014, **343**, 301–305.
- 94 G. Lu, R. E. Middleton, H. Sun, M. Naniong, C. J. Ott, C. S. Mitsiades, K.-K. Wong, J. E. Bradner and W. G. Kaelin, *Science*, 2014, **343**, 305–309.

- 95 E. S. Fischer, K. Böhm, J. R. Lydeard, H. Yang, M. B. Stadler, S. Cavadini, J. Nagel, F. Serluca, V. Acker, G. M. Lingaraju, R. B. Tichkule, M. Schebesta, W. C. Forrester, M. Schirle, U. Hassiepen, J. Ottl, M. Hild, R. E. J. Beckwith, J. W. Harper, J. L. Jenkins and N. H. Thomä, *Nature*, 2014, **512**, 49–53.
- 96 G. E. Winter, D. L. Buckley, J. Paulk, J. M. Roberts, A. Souza, S. Dhe-Paganon and J. E. Bradner, *Science*, 2015, **348**, 1376–1381.
- 97 L. N. Gechijian, D. L. Buckley, M. A. Lawlor, J. M. Reyes, J. Paulk, C. J. Ott, G. E. Winter, M. A. Erb, T. G. Scott, M. Xu, H.-S. Seo, S. Dhe-Paganon, N. P. Kwiatkowski, J. A. Perry, J. Qi, N. S. Gray and J. E. Bradner, *Nat. Chem. Biol.*, 2018, **14**, 405.
- 98 Z. I. Bassi, M. C. Fillmore, A. H. Miah, T. D. Chapman, C. Maller, E. J. Roberts, L. C. Davis, D. E. Lewis, N. W. Galwey, K. E. Waddington, V. Parravicini, A. L. Macmillan-Jones, C. Gongora, P. G. Humphreys, I. Churcher, R. K. Prinjha and D. F. Tough, *ACS Chem. Biol.*, 2018, **13**, 2862–2867.
- 99 J. Popow, H. Arnhof, G. Bader, H. Berger, A. Ciulli, D. Covini, C. Dank, T. Gmaschitz, P. Greb, J. Karolyi-Özguer, M. Koegl, D. B. McConnell, M. Pearson, M. Rieger, J. Rinnenthal, V. Roessler, A. Schrenk, M. Spina, S. Steurer, N. Trainor, E. Traxler, C. Wieshofer, A. Zoephel and P. Ettmayer, *J. Med. Chem.*, 2019, **62**, 2508–2520.
- 100 C. Zhang, X.-R. Han, X. Yang, B. Jiang, J. Liu, Y. Xiong and J. Jin, *Eur. J. Med. Chem.*, 2018, **151**, 304–314.
- 101 S. Liu, H. O. Yosief, L. Dai, H. Huang, G. Dhawan, X. Zhang, A. M. Muthengi, J. Roberts, D. L. Buckley, J. A. Perry, L. Wu, J. E. Bradner, J. Qi and W. Zhang, *J. Med. Chem.*, 2018, **61**, 7785–7795.
- 102 W. Li, C. Gao, L. Zhao, Z. Yuan, Y. Chen and Y. Jiang, *Eur. J. Med. Chem.*, 2018, **151**, 237–247.
- 103 M. Brand, B. Jiang, S. Bauer, K. A. Donovan, Y. Liang, E. S. Wang, R. P. Nowak, J. C. Yuan, T. Zhang, N. Kwiatkowski, A. C. Müller, E. S. Fischer, N. S. Gray and G. E. Winter, *Cell Chem. Biol.*, 2019, **26**, 300–306.e9.
- 104 V. Zoppi, S. J. Hughes, C. Maniaci, A. Testa, T. Gmaschitz, C. Wieshofer, M. Koegl, K. M. Riching, D. L. Daniels, A. Spallarossa and A. Ciulli, *J. Med. Chem.*, 2019, **62**, 699–726.
- 105 B. Zhou, J. Hu, F. Xu, Z. Chen, L. Bai, E. Fernandez-Salas, M. Lin, L. Liu, C.-Y. Yang, Y. Zhao, D. McEachern, S. Przybranowski, B. Wen, D. Sun and S. Wang, *J. Med. Chem.*, 2018, **61**, 462–481.
- 106 K. Yang, Y. Song, H. Xie, H. Wu, Y.-T. Wu, E. D. Leisten and W. Tang, *Bioorg. Med. Chem. Lett.*, 2018, **28**, 2493–2497.
- 107 M. Schiedel, D. Herp, S. Hammelmann, S. Swyter, A. Lehotzky, D. Robaa, J. Oláh, J. Ovádi, W. Sippl and M. Jung, *J. Med. Chem.*, 2018, **61**, 482–491.
- 108 C. Qin, Y. Hu, B. Zhou, E. Fernandez-Salas, C.-Y. Yang, L. Liu, D. McEachern, S. Przybranowski, M. Wang, J. Stuckey, J. Meagher, L. Bai, Z. Chen, M. Lin, J. Yang, D. N. Ziaadeh, F. Xu, J. Hu, W. Xiang, L. Huang, S. Li, B. Wen, D. Sun and S. Wang, *J. Med. Chem.*, 2018, **61**, 6685–6704.
- 109 J. Hu, B. Hu, M. Wang, F. Xu, B. Miao, C.-Y. Yang, M. Wang, Z. Liu, D. F. Hayes, K. Chinnaswamy, J. Delproposto, J. Stuckey and S. Wang, *J. Med. Chem.*, 2019, **62**, 1420–1442.
- 110 X. Han, C. Wang, C. Qin, W. Xiang, E. Fernandez-Salas, C.-Y. Yang, M. Wang, L. Zhao, T. Xu, K. Chinnaswamy, J. Delproposto, J. Stuckey and S. Wang, *J. Med. Chem.*, 2019, **62**, 941–964.
- 111 L. Wang, V. S. Guillen, N. Sharma, K. Flessa, J. Min, K. E. Carlson, W. Toy, S. Braqi, B. S. Katzenellenbogen, J. A. Katzenellenbogen, S. Chandarlapaty and A. Sharma, *ACS Med. Chem. Lett.*, 2018, **9**, 803–808.
- 112 W. McCoull, T. Cheung, E. Anderson, P. Barton, J. Burgess, K. Byth, Q. Cao, M. P. Castaldi, H. Chen, E. Chiarparin, R. J. Carbajo, E. Code, S. Cowan, P. R. Davey, A. D. Ferguson, S. Fillery, N. O. Fuller, N. Gao, D. Hargreaves, M. R. Howard, J. Hu, A. Kawatkar, P. D. Kemmitt, E. Leo, D. M. Molina, N. O'Connell, P. Petteruti, T. Rasmusson, P. Raubo, P. B. Rawlins, P. Ricchiuto, G. R. Robb, M. Schenone, M. J. Waring, M. Zinda, S. Fawell and D. M. Wilson, *ACS Chem. Biol.*, 2018, **13**, 3131–3141.
- 113 M. Lu, T. Liu, Q. Jiao, J. Ji, M. Tao, Y. Liu, Q. You and Z. Jiang, *Eur. J. Med. Chem.*, 2018, **146**, 251–259.
- 114 C. Steinebach, H. Kehm, S. Lindner, L. P. Vu, S. Köpff, Á. L. Mármol, C. Weiler, K. G. Wagner, M. Reichenzeller, J. Krönke and M. Gütschow, *Chem. Commun.*, 2019, **55**, 1821–1824.
- 115 Y. Li, J. Yang, A. Aguilar, D. McEachern, S. Przybranowski, L. Liu, C.-Y. Yang, M. Wang, X. Han and S. Wang, *J. Med. Chem.*, 2019, **62**, 448–466.
- 116 C. Maniaci, S. J. Hughes, A. Testa, W. Chen, D. J. Lamont, S. Rocha, D. R. Alessi, R. Romeo and A. Ciulli, *Nat. Commun.*, 2017, **8**, 830.
- 117 C. Steinebach, S. Lindner, N. D. Udeshi, D. C. Mani, H. Kehm, S. Köpff, S. A. Carr, M. Gütschow and J. Krönke, *ACS Chem. Biol.*, 2018, **13**, 2771–2782.
- 118 G. M. Burslem, B. E. Smith, A. C. Lai, S. Jaime-Figueroa, D. C. McQuaid, D. P. Bondeson, M. Toure, H. Dong, Y. Qian, J. Wang, A. P. Crew, J. Hines and C. M. Crews, *Cell Chem. Biol.*, 2018, **25**, 67–77.e3.
- 119 S. L. Fisher and A. J. Phillips, *Curr. Opin. Chem. Biol.*, 2018, **44**, 47–55.
- 120 E. F. Douglass, C. J. Miller, G. Sparer, H. Shapiro and D. A. Spiegel, *J. Am. Chem. Soc.*, 2013, **135**, 6092–6099.
- 121 M. J. Roy, S. Winkler, S. J. Hughes, C. Whitworth, M. Galant, W. Farnaby, K. Rumpel and A. Ciulli, *ACS Chem. Biol.*, 2019, **14**, 361–368.
- 122 M. S. Gadd, A. Testa, X. Lucas, K.-H. Chan, W. Chen, D. J. Lamont, M. Zengerle and A. Ciulli, *Nat. Chem. Biol.*, 2017, **13**, 514–521.
- 123 R. P. Nowak, S. L. DeAngelo, D. Buckley, Z. He, K. A. Donovan, J. An, N. Safaei, M. P. Jedrychowski, C. M. Ponthier, M. Ishoe, T. Zhang, J. D. Mancias, N. S. Gray, J. E. Bradner and E. S. Fischer, *Nat. Chem. Biol.*, 2018, **14**, 706–714.
- 124 C. P. Tinworth, H. Lithgow, L. Dittus, Z. I. Bassi, S. E. Hughes, M. Muelbaier, H. Dai, I. E. D. Smith, W. J. Kerr, G. A. Burley, M. Bantscheff and J. D. Harling, *ACS Chem. Biol.*, 2019, **14**, 342–347.
- 125 M. Zengerle, K.-H. Chan and A. Ciulli, *ACS Chem. Biol.*, 2015, **10**, 1770–1777.

- 126 D. P. Bondeson, B. E. Smith, G. M. Burslem, A. D. Buhimschi, J. Hines, S. Jaime-Figueroa, J. Wang, B. D. Hamman, A. Ishchenko and C. M. Crews, *Cell Chem. Biol.*, 2018, 25, 78–87.e5.
- 127 B. E. Smith, S. L. Wang, S. Jaime-Figueroa, A. Harbin, J. Wang, B. D. Hamman and C. M. Crews, *Nat. Commun.*, 2019, 10, 131.
- 128 N. E. A. Chessum, S. Y. Sharp, J. J. Caldwell, A. E. Pasqua, B. Wilding, G. Colombano, I. Collins, B. Ozer, M. Richards, M. Rowlands, M. Stubbs, R. Burke, P. C. McAndrew, P. A. Clarke, P. Workman, M. D. Cheeseman and K. Jones, *J. Med. Chem.*, 2018, 61, 918–933.
- 129 A. Mullard, *Nat. Rev. Drug Discovery*, 2019, 18, 237.
- 130 K. M. Riching, S. Mahan, C. R. Corona, M. McDougall, J. D. Vasta, M. B. Robers, M. Urh and D. L. Daniels, *ACS Chem. Biol.*, 2018, 13, 2758–2770.
- 131 M. L. Drummond and C. I. Williams, *J. Chem. Inf. Model.*, 2019, 59, 1634–1644.
- 132 S. Banik, K. Pedram, S. Wisnovsky, N. Riley and C. Bertozzi, *ChemRxiv*, DOI: 10.26434/chemrxiv.7927061.v1.
- 133 X. Fan, W. Y. Jin, J. Lu, J. Wang and Y. T. Wang, *Nat. Neurosci.*, 2014, 17, 471–480.
- 134 D. A. Nalawansha, S.-L. Paiva, D. N. Rafizadeh, M. Pettersson, L. Qin and C. M. Crews, *ACS Cent. Sci.*, 2019, 5, 1079–1084.
- 135 N. F. Rizvi and G. F. Smith, *Bioorg. Med. Chem. Lett.*, 2017, 27, 5083–5088.
- 136 G. W. Collie and G. N. Parkinson, *Chem. Soc. Rev.*, 2011, 40, 5867–5892.
- 137 C. Weldon, I. Behm-Ansmant, L. H. Hurley, G. A. Burley, C. Branlant, I. C. Eperon and C. Dominguez, *Nat. Chem. Biol.*, 2017, 13, 18–20.
- 138 D. N. Petersen, J. Hawkins, W. Ruangsiriluk, K. A. Stevens, B. A. Maguire, T. N. O'Connell, B. N. Rocke, M. Boehm, R. B. Ruggeri, T. Rolph, D. Hepworth, P. M. Loria and P. A. Carpino, *Cell Chem. Biol.*, 2016, 23, 1362–1371.
- 139 N. G. Lintner, K. F. McClure, D. Petersen, A. T. Londregan, D. W. Piotrowski, L. Wei, J. Xiao, M. Bolt, P. M. Loria, B. Maguire, K. F. Geoghegan, A. Huang, T. Rolph, S. Liras, J. A. Doudna, R. G. Dullea and J. H. D. Cate, *PLoS Biol.*, 2017, 15, e2001882.
- 140 N. F. Rizvi, J. A. Howe, A. Nahvi, D. J. Klein, T. O. Fischmann, H.-Y. Kim, M. A. McCoy, S. S. Walker, A. Hruza, M. P. Richards, C. Chamberlin, P. Saradjian, M. T. Butko, G. Mercado, J. Burchard, C. Strickland, P. J. Dandliker, G. F. Smith and E. B. Nickbarg, *ACS Chem. Biol.*, 2018, 13, 820–831.
- 141 N. Amodio, L. Raimondi, G. Juli, M. A. Stamato, D. Caracciolo, P. Tagliaferri and P. Tassone, *J. Hematol. Oncol.*, 2018, 11, 63.
- 142 A. Donlic, B. S. Morgan, J. L. Xu, A. Liu, C. Roble and A. E. Hargrove, *Angew. Chem., Int. Ed.*, 2018, 57, 13242–13247.
- 143 F. A. Abulwerdi, W. Xu, A. A. Ageeli, M. J. Yonkunas, G. Arun, H. Nam, J. S. Schneekloth, T. K. Dayie, D. Spector, N. Baird and S. F. J. Le Grice, *ACS Chem. Biol.*, 2019, 14, 223–235.
- 144 A. J. Angelbello, S. G. Rzuczek, K. K. McKee, J. L. Chen, H. Olafson, M. D. Cameron, W. N. Moss, E. T. Wang and M. D. Disney, *Proc. Natl. Acad. Sci. U. S. A.*, 2019, 116, 7799–7804.
- 145 K. D. Warner, C. E. Hajdin and K. M. Weeks, *Nat. Rev. Drug Discovery*, 2018, 17, 547–558.
- 146 L. Cong, F. A. Ran, D. Cox, S. Lin, R. Barretto, N. Habib, P. D. Hsu, X. Wu, W. Jiang, L. A. Marraffini and F. Zhang, *Science*, 2013, 339, 819–823.
- 147 M. Jinek, A. East, A. Cheng, S. Lin, E. Ma and J. Doudna, *eLife*, 2013, 2, e00471.
- 148 P. Mali, L. Yang, K. M. Esvelt, J. Aach, M. Guell, J. E. DiCarlo, J. E. Norville and G. M. Church, *Science*, 2013, 339, 823–826.
- 149 K. Karikó, H. Muramatsu, F. A. Welsh, J. Ludwig, H. Kato, S. Akira and D. Weissman, *Mol. Ther.*, 2008, 16, 1833–1840.
- 150 K. Elverum and M. Whitman, *Gene Ther.*, 2019, 1.
- 151 I. Lostalé-Seijo and J. Montenegro, *Nat. Rev. Chem.*, 2018, 2, 258–277.
- 152 A. B. Hill, M. Chen, C.-K. Chen, B. A. Pfeifer and C. H. Jones, *Trends Biotechnol.*, 2016, 34, 91–105.
- 153 L. A. Gilbert, M. H. Larson, L. Morsut, Z. Liu, G. A. Brar, S. E. Torres, N. Stern-Ginossar, O. Brandman, E. H. Whitehead, J. A. Doudna, W. A. Lim, J. S. Weissman and L. S. Qi, *Cell*, 2013, 154, 442–451.
- 154 U. Sahin, K. Karikó and Ö. Türeci, *Nat. Rev. Drug Discovery*, 2014, 13, 759–780.
- 155 L. Warren, P. D. Manos, T. Ahfeldt, Y.-H. Loh, H. Li, F. Lau, W. Ebina, P. K. Mandal, Z. D. Smith, A. Meissner, G. Q. Daley, A. S. Brack, J. J. Collins, C. Cowan, T. M. Schlaeger and D. J. Rossi, *Cell Stem Cell*, 2010, 7, 618–630.
- 156 L. Zangi, K. O. Lui, A. von Gise, Q. Ma, W. Ebina, L. M. Ptaszek, D. Später, H. Xu, M. Tabebordbar, R. Gorbato, B. Sena, M. Nahrendorf, D. M. Briscoe, R. A. Li, A. J. Wagers, D. J. Rossi, W. T. Pu and K. R. Chien, *Nat. Biotechnol.*, 2013, 31, 898–907.
- 157 L. Carlsson, J. C. Clarke, C. Yen, F. Gregoire, T. Albery, M. Billger, A.-C. Egnell, L.-M. Gan, K. Jennbacken, E. Johansson, G. Linhardt, S. Martinsson, M. W. Sadiq, N. Witman, Q.-D. Wang, C.-H. Chen, Y.-P. Wang, S. Lin, B. Ticho, P. C. H. Hsieh, K. R. Chien and R. Fritsche-Danielson, *Mol. Ther.–Methods Clin. Dev.*, 2018, 9, 330–346.
- 158 E. Robinson, K. D. MacDonald, K. Slaughter, M. McKinney, S. Patel, C. Sun and G. Sahay, *Mol. Ther.*, 2018, 26, 2034–2046.
- 159 R. S. Riley, C. H. June, R. Langer and M. J. Mitchell, *Nat. Rev. Drug Discovery*, 2019, 18, 175.
- 160 A. K. Patel, J. C. Kaczmarek, S. Bose, K. J. Kauffman, F. Mir, M. W. Heartlein, F. DeRosa, R. Langer and D. G. Anderson, *Adv. Mater.*, 2019, 31, 1805116.
- 161 L.-M. Gan, M. Lagerström-Fermér, L. G. Carlsson, C. Arfvidsson, A.-C. Egnell, A. Rudvik, M. Kjaer, A. Collén, J. D. Thompson, J. Joyal, L. Chialda, T. Koernicke, R. Fuhr, K. R. Chien and R. Fritsche-Danielson, *Nat. Commun.*, 2019, 10, 871.
- 162 D. Adams, A. Gonzalez-Duarte, W. D. O'Riordan, C.-C. Yang, M. Ueda, A. V. Kristen, I. Tournev, H. H. Schmidt, T.

- Coelho, J. L. Berk, K.-P. Lin, G. Vita, S. Attarian, V. Planté-Bordeneuve, M. M. Mezei, J. M. Campistol, J. Buades, T. H. Brannagan, B. J. Kim, J. Oh, Y. Parman, Y. Sekijima, P. N. Hawkins, S. D. Solomon, M. Polydefkis, P. J. Dyck, P. J. Gandhi, S. Goyal, J. Chen, A. L. Strahs, S. V. Nochur, M. T. Sweetser, P. P. Garg, A. K. Vaishnav, J. A. Gollob and O. B. Suhr, *N. Engl. J. Med.*, 2018, **379**, 11–21.
- 163 L.-C. Li, S. T. Okino, H. Zhao, D. Pookot, R. F. Place, S. Urakami, H. Enokida and R. Dahiya, *Proc. Natl. Acad. Sci. U. S. A.*, 2006, **103**, 17337–17342.
- 164 J. Voutila, V. Reebye, T. C. Roberts, P. Protopapa, P. Andrikakou, D. C. Blakey, R. Habib, H. Huber, P. Saetrom, J. J. Rossi and N. A. Habib, *Mol. Ther.*, 2017, **25**, 2705–2714.
- 165 R. Rupaimoole and F. J. Slack, *Nat. Rev. Drug Discovery*, 2017, **16**, 203–222.
- 166 C. F. Bennett and E. E. Swayze, *Annu. Rev. Pharmacol. Toxicol.*, 2010, **50**, 259–293.
- 167 L. Guan and M. D. Disney, *ACS Chem. Biol.*, 2012, **7**, 73–86.
- 168 M. G. Costales, C. L. Haga, S. P. Velagapudi, J. L. Childs-Disney, D. G. Phinney and M. D. Disney, *J. Am. Chem. Soc.*, 2017, **139**, 3446–3455.
- 169 L. Guan and M. D. Disney, *Angew. Chem., Int. Ed.*, 2013, **52**, 10010–10013.
- 170 M. G. Costales, Y. Matsumoto, S. P. Velagapudi and M. D. Disney, *J. Am. Chem. Soc.*, 2018, **140**, 6741–6744.
- 171 Y. Li and M. D. Disney, *ACS Chem. Biol.*, 2018, **13**, 3065–3071.
- 172 A. G. Matera and Z. Wang, *Nat. Rev. Mol. Cell Biol.*, 2014, **15**, 108–121.
- 173 J. Palacino, S. E. Swalley, C. Song, A. K. Cheung, L. Shu, X. Zhang, M. Van Hoosear, Y. Shin, D. N. Chin, C. G. Keller, M. Beibel, N. A. Renaud, T. M. Smith, M. Salcius, X. Shi, M. Hild, R. Servais, M. Jain, L. Deng, C. Bullock, M. McLellan, S. Schuierer, L. Murphy, M. J. J. Blommers, C. Blaustein, F. Berenshteyn, A. Lacoste, J. R. Thomas, G. Roma, G. A. Michaud, B. S. Tseng, J. A. Porter, V. E. Myer, J. A. Tallarico, L. G. Hamann, D. Curtis, M. C. Fishman, W. F. Dietrich, N. A. Dales and R. Sivasankaran, *Nat. Chem. Biol.*, 2015, **11**, 511–517.
- 174 N. A. Naryshkin, M. Weetall, A. Dakka, J. Narasimhan, X. Zhao, Z. Feng, K. K. Y. Ling, G. M. Karp, H. Qi, M. G. Woll, G. Chen, N. Zhang, V. Gabbeta, P. Vazirani, A. Bhattacharyya, B. Furia, N. Risher, J. Sheedy, R. Kong, J. Ma, A. Turpoff, C.-S. Lee, X. Zhang, Y.-C. Moon, P. Trifillis, E. M. Welch, J. M. Colacino, J. Babiak, N. G. Almstead, S. W. Peltz, L. A. Eng, K. S. Chen, J. L. Mull, M. S. Lynes, L. L. Rubin, P. Fontoura, L. Santarelli, D. Haehnke, K. D. McCarthy, R. Schmucki, M. Ebeling, M. Sivaramakrishnan, C.-P. Ko, S. V. Paushkin, H. Ratni, I. Gerlach, A. Ghosh and F. Metzger, *Science*, 2014, **345**, 688–693.
- 175 R. S. Finkel, E. Mercuri, B. T. Darras, A. M. Connolly, N. L. Kuntz, J. Kirschner, C. A. Chiriboga, K. Saito, L. Servais, E. Tizzano, H. Topaloglu, M. Tulinius, J. Montes, A. M. Glanzman, K. Bishop, Z. J. Zhong, S. Gheuens, C. F. Bennett, E. Schneider, W. Farwell and D. C. De Vivo, *N. Engl. J. Med.*, 2017, **377**, 1723–1732.
- 176 A. K. Cheung, B. Hurley, R. Kerrigan, L. Shu, D. N. Chin, Y. Shen, G. O'Brien, M. J. Sung, Y. Hou, J. Axford, E. Cody, R. Sun, A. Fazal, C. Fridrich, C. C. Sanchez, R. C. Tomlinson, M. Jain, L. Deng, K. Hoffmaster, C. Song, M. Van Hoosear, Y. Shin, R. Servais, C. Towler, M. Hild, D. Curtis, W. F. Dietrich, L. G. Hamann, K. Briner, K. S. Chen, D. Kobayashi, R. Sivasankaran and N. A. Dales, *J. Med. Chem.*, 2018, **61**, 11021–11036.
- 177 H. Ratni, M. Ebeling, J. Baird, S. Bendels, J. Bylund, K. S. Chen, N. Denk, Z. Feng, L. Green, M. Guerard, P. Jablonski, B. Jacobsen, O. Khwaja, H. Kletzl, C.-P. Ko, S. Kustermann, A. Marquet, F. Metzger, B. Mueller, N. A. Naryshkin, S. V. Paushkin, E. Pinard, A. Poirier, M. Reutlinger, M. Weetall, A. Zeller, X. Zhao and L. Mueller, *J. Med. Chem.*, 2018, **61**, 6501–6517.
- 178 W. V. Gilbert, T. A. Bell and C. Schaening, *Science*, 2016, **352**, 1408–1412.
- 179 Y. Saletore, K. Meyer, J. Korlach, I. D. Vilfan, S. Jaffrey and C. E. Mason, *Genome Biol.*, 2012, **13**, 175.
- 180 P. A. Boriack-Sjodin, S. Ribich and R. A. Copeland, *Nat. Rev. Drug Discovery*, 2018, **17**, 435–453.
- 181 X. Li, X. Xiong and C. Yi, *Nat. Methods*, 2017, **14**, 23–31.
- 182 L. P. Vu, B. F. Pickering, Y. Cheng, S. Zaccara, D. Nguyen, G. Minuesa, T. Chou, A. Chow, Y. Saletore, M. MacKay, J. Schulman, C. Famulare, M. Patel, V. M. Klimek, F. E. Garrett-Bakelman, A. Melnick, M. Carroll, C. E. Mason, S. R. Jaffrey and M. G. Kharas, *Nat. Med.*, 2017, **23**, 1369–1376.
- 183 M. Schapira, *ACS Chem. Biol.*, 2016, **11**, 575–582.
- 184 P. J. McEnaney, C. G. Parker and A. X. Zhang, in *Annual Reports in Medicinal Chemistry*, ed. R. A. Goodnow, Academic Press, 2017, vol. 50, pp. 481–518.
- 185 K. Ito, K. Sakai, Y. Suzuki, N. Ozawa, T. Hatta, T. Natsume, K. Matsumoto and H. Suga, *Nat. Commun.*, 2015, **6**, 6373.
- 186 A. T. Plowright, C. Ottmann, M. Arkin, Y. P. Auberson, H. Timmerman and H. Waldmann, *Cell Chem. Biol.*, 2017, **24**, 1058–1065.