Cyanobacteria have attracted much attention as hosts to recycle CO₂ into valuable chemicals. Although cyanobacteria have been engineered to produce various compounds, production efficiencies are too low for commercialization. Here we engineer the carbon metabolism of *Synechococcus elongatus* PCC 7942 to improve glucose utilization, enhance CO₂ fixation and increase chemical production. We introduce modifications in glycolytic pathways and the Calvin Benson cycle to increase carbon flux and redirect it towards carbon fixation. The engineered strain efficiently uses both CO₂ and glucose, and produces 12.6 g l⁻¹ of 2,3-butanediol with a rate of 1.1 g l⁻¹ d⁻¹ under continuous light conditions. Removal of native regulation enables carbon fixation and 2,3-butanediol production in the absence of light. This represents a significant step towards industrial viability and an excellent example of carbon metabolism plasticity.
Metabolic engineering of photosynthetic organisms allows solar energy to power carbon capture and the production of food, fuels and valuable chemicals\(^{1,2}\). Cyanobacteria have attracted much interest as hosts for photosynthetic chemical production due to the simplicity of culture conditions, ease of genetic manipulation and relatively fast cell growth compared to higher plants\(^{3-6}\). Several cyanobacterial strains have been engineered for photosynthetic chemical production. However, despite progress in metabolic manipulation and analysis, titres and productivities from cyanobacteria are still far below industrial feasibility\(^{7,8}\).

Dependency on continuous lighting and the slow process of carbon fixation are particularly limiting\(^{9,10}\). We have previously engineered the model cyanobacterium *Synechococcus elongatus* PCC 7942 to produce 2,3-butanediol (23BD) from CO\(_2\) and glucose\(^{11-13}\). Under natural light, chemical production from an engineered photosynthetic organism would be confined to a limited window of optimal sunlight exposure\(^{13}\). However, in order to achieve industrial feasibility, chemical production under both light and dark conditions is essential, as demonstrated in our previous work, concurrent expression of heterologous sugar importers and the 23BD biosynthetic pathway genes allows for chemical production and growth from both CO\(_2\) and glucose\(^{13}\). The oxidation of sugars provides an increased supply of metabolites and energy independent of photosynthesis, allowing for faster cell growth and continuous chemical production throughout diurnal conditions (12 h light/12 h dark).

Carbon yield is one of the most important factors for economic feasibility and is calculated based on the theoretical maximum yield (TMY). In this study, two substrates, CO\(_2\) and glucose, are utilized simultaneously. Owing to their concurrent use and culturing conditions that allow for gas exchange with the environment, it is impossible to directly measure TMY from both substrates. Therefore, we evaluate photomixotrophic production by calculating TMY possible from glucose alone (0.5 \(\mu\)g 23BD/\(\mu\)g glucose), TMY values above 100% indicate 23BD production beyond what is possible from glucose alone, and we assume that these yields reflect the incorporation of CO\(_2\) in addition to glucose. It is important to note that glucose utilization is not 100% efficient. The value of the TMY minus the maximum contribution from glucose (100%) represents only the minimum value for CO\(_2\) incorporation, and the true contribution is likely higher.

The carbon yield achieved in our previous study was 40% of TMY\(^{12}\), with great potential for improvement. Thus, the goal of this study is to optimize glucose and CO\(_2\) utilization and to improve 23BD production and yield. To accomplish this, we identify and relieve bottlenecks in glucose catabolism, deregulate and enhance CO\(_2\) fixation, combine successful modifications and identify and relieve bottlenecks in glucose catabolism, deregulate and enhance CO\(_2\) fixation, combine successful modifications and improve 23BD production and yield. To accomplish this, we success\(^{15,16}\). Several studies have shown that the catalytic efficiency of RuBisCO is already naturally optimized\(^{17,18}\).

Rather than focusing on improving RubisCO, we examine carbon metabolism as a whole. Enzyme and metabolite pools of the regenerative phase of the CB cycle are proposed to play a role in determining the overall carbon fixation rate\(^{19,20}\). Modifications of other steps in the CB cycle, such as those catalysed by sedoheptulose 1,7-bisphosphatase and transketolase, have been utilized to improve carbon fixation\(^{19-21}\). Therefore, we hypothesize that the supplemental carbon source glucose would increase availability of the substrate for carbon fixation, D-ribulose-1,5-bisphosphate (R15P), and enhance efficiency of the reaction catalysed by RuBisCO.

Here we propose a strategy to increase carbon fixation and chemical production in cyanobacterial grown under light and dark conditions. First, glucose metabolism is rewired through the oxidative pentose phosphate (OPP) pathway to overproduce ribulose-5-phosphate (Ru5P), a precursor of the CO\(_2\) fixation pathway. Next, because carbon metabolism in cyanobacteria is precisely controlled in response to environmental changes such as light and carbon availability\(^{22-24}\), *cp12*, a regulatory gene of the CB cycle, is deleted to amplify conversion of Ru5P to R15P (Fig. 1a,b). This integrated approach of essential gene overexpression and deletion of *cp12* allows for enhanced CO\(_2\) fixation, a remarkable increase of 23BD production in both light and dark conditions through light-independent supply of glucose carbons (Fig. 1a,b), and could be applied as a general strategy for improving the efficiency of other photosynthetic organisms.

### Results

**Characterization of glucose catabolism in engineered strains.** To achieve efficient 23BD production from glucose and CO\(_2\), we first optimized the genetic construct for the 23BD biosynthesis pathway (Fig. 1c), then paired this optimization with expression of *galP*, which encodes a galactose-proton symporter from *Escherichia coli* (Strain 3, Table 1). Details of optimization and a summary of our previous work on 23BD production in *S. elongatus* PCC 7942 can be found in the Supplementary Figs 1, and 2 and Supplementary Note 1. Strain 3 produced 2.5 g l\(^{-1}\) of 23BD when glucose was supplied, whereas only 0.4 g l\(^{-1}\) was produced without glucose (Fig. 2a,b).

To investigate how glucose affects the carbon metabolism of Strain 3, we applied metabolomics analysis. Quantification of metabolites from Strain 3 may explain how the flow of carbon changes in *S. elongatus* because of glucose, which may in turn allow optimization of carbon metabolism and 23BD production of the engineered strains. Cells were grown with or without glucose for 72 h under continuous light. Among the several hundred metabolite signals detected, 136 metabolites were identified and quantified\(^{25,26}\) (Fig. 1c and Supplementary Data 1) and some key changes to central carbon pathway metabolites were observed. Levels of gluconate and fructose-6-phosphate (F6P) increased by 7.3- and 4.1-fold, respectively, in the presence of glucose (Fig. 1c). In contrast, metabolites of the lower part of the Embden–Meyerhof–Parnas (EMP) pathway, 3-phosphoglycerate (3PGA) and phosphoenolpyruvate (PEP) were lowered by 66% and 88%, respectively, upon the addition of glucose (Fig. 1c). This has also been observed in *Synechocystis* sp. PCC 6803 under similar conditions\(^{27}\). Moreover, a decrease of TCA cycle metabolites, including malate, fumarate, succinate and citrate, was observed in glucose-fed cells (Fig. 1c).

In order to understand these data further, we constructed several deletion mutants from Strain 3 (Table 1) that would separately inactivate one of the branches of carbon metabolism. Three main pathways for glucose catabolism in cyanobacteria were investigated: the OPP pathway, the EMP pathway and the Entner–Doudoroff (ED) pathway (Fig. 1c). Genes encoding the following enzymes were chosen as deletion targets in order to assess the metabolic contribution of each pathway: glucose-6-phosphate dehydrogenase (encoded by zwf, Strain 4) and 6-phosphogluconate (6PG) dehydrogenase (*gnd*, Strain 5) of the OPP pathway, phosphoglucone isomerase (*pgi*, Strain 6) and phosphofructokinase (*pfk*, Strain 7) of the EMP pathway, and 2-keto-3-deoxygluconate 6-phosphate aldolase (*eda*, Strain 8) of the ED pathway (Table 1). Gene replacement with an antibiotic...
resistance gene and complete segregation of Strains 4 ($\Delta$zwf) and 7 ($\Delta$pfk) were verified by PCR and sequencing. However, despite repeated trials, complete deletion of gnd, pgi and eda from the genome could not be achieved, indicating that these genes were essential in our culture conditions. However, we chose to include these strains in further tests to characterize the effects of partial knockouts of each gene. It is of note, however, that gnd has been successfully deleted in wild-type S. elongatus28,29. We expected that disruption of pathways responsible for glucose metabolism would result in a defect in growth and/or 23BD production. The photomixotrophic growth of Strain 4 ($\Delta$zwf) was impaired after 72 h (Fig. 2a). Also, 23BD production of Strain 4 decreased by 62% compared to Strain 3 when grown with glucose (Fig. 2b). However, other mutants showed no significant changes in biomass or 23BD production compared to Strain 3 (Fig. 2a,b). Impairment of growth and 23BD production of Strain 4 suggested that glucose is metabolized through the OPP pathway, but if that were the case, then we would expect Strain 5 to also display defects. That Strain 5 (or Strains 6–8) did not show growth or 23BD production defects makes it uncertain which of the three pathways was primarily responsible for metabolizing fed glucose. Also, it is possible that pathway disruption was compensated for by upregulation of other glucose metabolism pathways. Therefore, we decided to individually overexpress genes of all three pathways in order to test their influence on 23BD production.

Guiding glucose flux in central carbon metabolism. To determine which pathway is responsible for glucose metabolism, and to explore the benefits of directed glucose flux, we overexpressed the key genes of each pathway (Strains 9, gatP-zwf-edd (ED); 10, galP-pgi (EMP); 11, galP-zwf-gnd (OPP), Table 1 and Supplementary Fig. 2). In vitro enzyme assays confirmed the functional expression of each genes (Supplementary Table 4). Enzymatic activities of ZWF and GND were 14- and 3.6-fold higher, respectively, in Strain 11 than in Strain 3 (without overexpression) (Supplementary Table 4). Strain 10 showed a large increase in activity of PGI, whereas no significant activity was detected in Strain 3 (Supplementary Table 4). Therefore, in vivo experiments were conducted to test the effects of these overexpressed genes on growth and 23BD production. Strains were grown with the addition of glucose under continuous light for 72 h (Fig. 2c–e). We expected an increase in growth, 23BD production and/or increased glucose consumption to be correlated with overexpression of an active glucose metabolism...
glucose concentration (NaHCO₃ in continuous light conditions. IPTG (0.1 mM) was added at 0 h. Cell growth (47NATURE COMMUNICATIONS | DOI: 10.1038/ncomms14724 | www.nature.com/naturecommunications

Figure 2 | Characterization and activation of glucose metabolism. Cells were cultured in 10 ml of BG11 media containing 10 g l⁻¹ glucose and 20 mM NaHCO₃ in continuous light conditions. IPTG (0.1 mM) was added at 0 h. Cell growth (a) and 23BD concentration (b) profiles of Strains 3 (black), 4 (Δzwf, red), 5 (gnd/Δgnd, green), 6 (pgi/Δpgi, blue), 7 (Δpk, purple) and 8 (eda/Δeda, orange). (c-e) Cell growth (c), 23BD concentration (d) and glucose concentration (e) profiles of Strains 3 (grey), 9 (galP-zwf-edd, green), 10 (galP-pgi, blue) and 11 (galP-zwf-gnd, red). N = 3 biological replicates; error bars represent s.d.

Redirection of carbon flux towards 23BD biosynthesis. We also wanted to explore whether modification to the CB cycle and its regulation would be beneficial for our production strain. Strain 9 (galP-zwf-edd) showed similar growth and glucose consumption rates compared to Strain 3 (galP), but decreased 23BD production (Fig. 2c–e). By contrast, the growth rates of Strains 10 (galP-pgi) and 11 (galP-zwf-gnd) were enhanced by 87% and 82%, respectively (Fig. 2c), and the glucose consumption rates of these strains increased by 118% and 129%, respectively, compared to Strain 3 (Fig. 2e). This provides strong evidence that glucose is metabolized through both the EMP and the OPP pathways. However, the 23BD production of Strains 10 and 11 was reduced compared to that of Strain 3 (Fig. 2d), suggesting that a larger portion of carbon flux from glucose was utilized for biomass formation rather than 23BD production in Strains 10 and 11. To improve glucose consumption by the ED pathway, we modified Strain 9 by additionally expressing the downstream gene, eda, resulting in Strain 9–2. Similarly, we expressed pfkA from the EMP pathway, resulting in Strain 10–2 (Table 1). However, neither glucose consumption nor 23BD production was improved in Strains 9–2 and 10–2 compared to the parent strains (Supplementary Fig. 3). Because glucose metabolism was improved in Strains 10 and 11 (Fig. 2e), we next focused on redirecting carbon flux towards 23BD production rather than biomass formation.

Direction of carbon flux towards 23BD biosynthesis. We also wanted to explore whether modification to the CB cycle and its regulation would be beneficial for our production strain.

Table 1 | Strains used in this study.

| Strains   | Strain No. | Genotype | References |
|-----------|------------|----------|------------|
| AL257     | AL257      | Synechococcus elongatus PCC 7942 | Golden 43 |
| AL2491    | AL257 + NSII: lacR; Ptrc: alsD-alsS-adh; gentR | This study |
| AL2253    | AL257 + NSII: lacR; Ptrc: alsD-alsS-adh; gentR | 47 |
| AL2456    | 3 + zwf: kanR | This study |
| AL2799    | 4 + zwf: kanR | This study |
| AL2800    | 5 + gnd/Δgnd: kanR | This study |
| AL2798    | 6 + gnd/Δgnd: kanR | This study |
| AL2680    | 7 + pfk: cmR | This study |
| AL2801    | 8 + eda/Δeda: kanR | This study |
| AL2894    | 9 + NSI: lacR; Ptrl: galP-zwf-edd; specR | This study |
| AL2937    | 9–2 + NSI: lacR; Ptrl: eda; kanR | This study |
| AL2895    | 10 + NSI: lacR; Ptrl: galP-pgi; specR | This study |
| AL2936    | 10–2 + NSI: lacR; Ptrl: pfkA; kanR | This study |
| AL2896    | 11 + NSI: lacR; Ptrl: galP-zwf-gnd; specR | This study |
| AL2556    | 12 + cp12: cmR | This study |
| AL2568    | 13 + cp12: lacR; Ptrl: rbcLXS; kanR | This study |
| AL2847    | 14 + cp12: lacR; Ptrl: prk-rbcLXS; kanR | This study |
| AL2935    | 15 + cp12: lacR; Ptrl: prk-rbcLXS; kanR | This study |
| AL1793    | 16 + AL257 + NSII: lacR; Ptrl: sfgfp; gentR | 47 |
| AL2575    | 17 + AL257 + NSII: lacR; Ptrl: sfgfp; gentR | This study |

Figure 2 | Characterization and activation of glucose metabolism. Cells were cultured in 10 ml of BG11 media containing 10 g l⁻¹ glucose and 20 mM NaHCO₃ in continuous light conditions. IPTG (0.1 mM) was added at 0 h. Cell growth (a) and 23BD concentration (b) profiles of Strains 3 (black), 4 (Δzwf, red), 5 (gnd/Δgnd, green), 6 (pgi/Δpgi, blue), 7 (Δpk, purple) and 8 (eda/Δeda, orange). (c–e) Cell growth (c), 23BD concentration (d) and glucose concentration (e) profiles of Strains 3 (grey), 9 (galP-zwf-edd, green), 10 (galP-pgi, blue) and 11 (galP-zwf-gnd, red). N = 3 biological replicates; error bars represent s.d.

Redirection of carbon flux towards 23BD biosynthesis. We also wanted to explore whether modification to the CB cycle and its regulation would be beneficial for our production strain.
Specifically, we sought to direct more carbon flux through the CB cycle by overexpressing crucial genes of the pathway and removing native regulation of the cycle. CP12 is a small regulatory protein that represses two important enzymes of the CB cycle, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and phosphoribulokinase (PRK). We hypothesized that the deletion of cp12 could be effective for redirecting the carbon flux through the CB cycle towards 23BD production. CP12 is activated in response to an elevated NAD(H)/NADP(H) ratio, a condition that was previously observed in photomixotrophic27 and dark22 conditions. Once activated, CP12 inhibits GAPDH, which controls the carbon flux towards the lower EMP pathway in cyanobacteria30. CP12 also inhibits PRK, which is responsible for the conversion of Ru5P to R15P, the substrate for the CO2 fixation by RuBisCO (Fig. 1c). Thus, the deletion of cp12 should increase carbon flux to the CB cycle, and may increase the rate of CO2 fixation, both of which should increase the intracellular pool of pyruvate and lead to an improvement in 23BD production.

To test whether changing regulation of carbon metabolism could increase carbon flow to 23BD biosynthesis, we constructed three strains. We disrupted the cp12 gene in Strain 3 to understand whether the absence of cp12 alone is sufficient for metabolic flux redirection. We also disrupted cp12 and inserted one of two sets of essential genes involved in carbon fixation: rbcL-rbcX-rbcS, which encodes the RuBisCO subunits and their chaperone from Synechococcus sp. PCC 7002, and rbcLXS plus prk, which encodes PRK from S. elongatus (Supplementary Fig. 2). Despite multiple attempts, a strain with Δcp12:: prk and no additional rbcLXS could not be successfully constructed. Synechococcus sp. PCC 7002 is known as one of the fastest-growing cyanobacteria31; therefore, we hypothesized that this particular RuBisCO would show desirable enzyme kinetics. We expected that overexpression of rbcLXS and prk independent of native regulation would increase carbon flow through these bottleneck steps of the CB cycle, providing additional carbon for 23BD synthesis. These modifications resulted in Strains 12 (3 + Δcp12), 13 (3 + ΔrbcLXS: rbcLXS) and 14 (3 + ΔrbcLXS: prk-rbcLXS; Table 1). Amplified activity of PRK (1.3-fold) was confirmed in Strain 14 compared to Strain 3; however, there was no significant difference in RuBisCO activity between Strain 13 or 14 and Strain 3 (Supplementary Table 4).

We hypothesized that these strains would result in improvements in 23BD production by increasing CO2 incorporation. Thus, 13C-labelled substrates were used to observe differences in the ratio of 23BD derived from CO2 versus fed glucose. Strains 3, 12, 13 and 14 were monitored for 23BD production after growth with 10 g l-1 of U-13C glucose and 20 mM unlabelled NaHCO3 for 24 h under continuous dark conditions.13C labelling ratio of intracellular 3PGA (d) and concentration of intracellular 15R15P normalized with that of Strain 12 (e). N = 3 biological replicates; error bars represent s.d. N.D. indicates not detectable.

To test our hypothesis that these strains could grow and produce 23BD independent of light, Strains 3, 12 (Δcp12) and 14 (Δcp12: prk-rbcLXS) were grown with 10 g l-1 glucose and 20 mM 13C-NaHCO3 in complete darkness for 24 h, and the 13C ratio of intracellular 3PGA was determined. (Strain 13 was not tested since it showed very little improvement of carbon fixation compared to Strains 12 and 14.) 3PGA is a direct product of the reaction catalysed by RuBisCO, and so labelled 3PGA would be clear evidence of carbon fixation. In the 3PGA from Strain 14, 12.6% of the carbons were labelled with 13C. Very few carbons were labelled in Strain 12 and no labelled carbons were detected in Strain 3 (Fig. 3d). These results indicate that deletion of cp12 permitted limited CO2 fixation in darkness, and deletion of cp12 combined with overexpression of prk (and rbcLXS) resulted in substantial CO2 fixation by RuBisCO in darkness. As further evidence of enhanced carbon flux to the CO2 fixation pathway, we also observed a 1.5-fold increase in the intracellular concentration of the RuBisCO substrate, R15P, in Strain 14 compared to Strain 12, and no accumulation of R15P was observed in Strain 3.
Coupling glucose metabolism and CO₂ fixation. With the success of our engineering strategies for increased glucose consumption (by overexpression of the EMP or the OPP pathway, Strains 10 (galP-pgi) and 11 (galP-zwf-gnd)), and increased carbon fixation (by deletion of cp12 and overexpression of prk and rbcLXS, Strain 14 (Δcp12:: prk-rbcLXS)), we hypothesized that a combination of these would produce a synergistic effect to further improve 23BD production and CO₂ fixation. Since the OPP pathway is the most direct route from glucose to 23BD production observed in Strain 14 (galP-pgi), we replaced cp12 with prk and rbcLXS (Strain 15, Table 1). Strains 3 (galP), 11 (galP-zwf-gnd), 14 (3 + Δcp12:: prk-rbcLXS) and 15 (11 + Δcp12:: prk-rbcLXS) were cultured with 10 g l⁻¹ glucose and 20 mM NaHCO₃ for 48 h, and growth, glucose consumption and 23BD production were quantified (Fig. 4). The growth and glucose consumption rates of Strains 11 and 15 were roughly three times higher than those of Strains 3 and 14 (Fig. 4a,c), and a remarkable improvement of 23BD production (1.4 g l⁻¹ at 48 h compared to 0.5 g l⁻¹ in Strain 3) was observed in Strain 15 (Fig. 4b).

To analyse the mechanism of the drastic improvement of 23BD production observed in Strain 15 (galP-zwf-gnd + Δcp12:: prk-rbcLXS), metabolomics analysis was applied to Strains 3, 11 and 15 (Fig. 4d, Supplementary Data 2 and Supplementary Fig. 4). Gluconate, 3PGA and PEP were markedly reduced in Strains 11 and 15 compared to Strain 3, whereas a reduction of G6P, F6P, Ru5P and ribose-5-phosphate was only observed in Strain 15 (Fig. 4d). These data suggest that carbon flow through these steps of glucose metabolism towards the CB cycle has been streamlined by overexpression of the OPP pathway in Strain 15. In particular, the large reduction (~90%) of Ru5P in Strain 15 compared to Strains 3 and 11 suggests that carbons were successfully redirected to the carbon fixation pathway by the action of PRK. Also, the significant increase of R15P, the substrate of CO₂ fixation, in Strain 15 (Fig. 4e) suggests that RubisCO is the bottleneck in an otherwise streamlined metabolism of glucose. It would be useful to quantify carbon flux as a way to directly compare the carbon fixation efficiency of these engineered strains. 23BD is easily quantified; however, because of the decarboxylation steps in the 23BD biosynthesis pathway, 23BD is not an accurate measure of newly fixed carbon (Supplementary Fig. 5). Therefore, the intracellular concentration of R15P was determined to evaluate the relative carbon flux in the carbon fixation pathway. It is very likely that the concentrations of Ru5P and R15P were lower than the Km values of PRK (270 µM; ref. 32) and RubisCO (27 µM; ref. 33), respectively. Thus, the increase of R15P indicates an enhanced carbon flux in the pathway. R15P content of Strain 15 (galP-zwf-gnd + Δcp12:: prk-rbcLXS) was increased by 4.6-fold compared to Strain 3 (galP; Fig. 4e). By contrast, there was no significant difference between Strains 3 and 11 (galP-zwf-gnd). In addition, specific production of

Figure 4 | Coupling the OPP pathway and the CO₂ fixation pathway. Cells were cultured in 10 ml of BG11 media containing 10 g l⁻¹ glucose and 20 mM NaHCO₃ in continuous light conditions (a-e) and dark conditions (f-g). OPP = with (+ + +) and without (+) overexpression of zwf and gnd; CP12 = cp12 gene was intact (+) and deleted (−) with prk and rbcLXS. Metabolite abbreviations are the same as those used in Fig. 1. Growth (a), 23BD concentration (b) and glucose concentration (c) profiles of Strains 3 (grey), 11 (galP-zwf-gnd, blue), 14 (Δcp12:: prk-rbcLXS, brown) and 15 (11 + Δcp12:: prk-rbcLXS, red). Intracellular concentrations of metabolites (d) of Strain 3, 11 and 15 at 48 h. Relative content of intracellular R15P of Strains 3, 11, 14 and 15 grown in continuous light (e) and dark (f) conditions for 24 h. Cell biomass and 23BD production (g) of the same strains grown in continuous dark conditions for 24 h. N = 3 biological replicates; error bars represent s.d. N.D. indicates not detectable.
intracellular alanine from CO₂ was examined by feeding ¹³C-NaHCO₃ and unlabelled glucose. Alanine is generated from pyruvate by a single reaction catalysed by alanine transaminase. Thus, alanine’s intracellular pool size can be correlated to carbon flux towards pyruvate and 23BD productivity. An increase of twofold in ¹³C-labelled alanine was observed in Strain 15 compared to Strains 3 and 11 (Supplementary Fig. 6), supporting the conclusion that carbon flux through the CB pathway was enhanced.

Notable changes in metabolites related to biomass formation were also observed that may at least partially explain the increased 23BD production of Strain 15. A reduction of metabolites required for purine and pyrimidine biosynthesis, such as adenine and thymidine 5-phosphate (dTTP), and those required for lipid production, such as glycerol-3-phosphate, was observed in Strain 15 (Supplementary Fig. 4) but not in Strain 11. Since these metabolites are associated with biomass formation, their decrease may reflect a change in carbon partitioning between Strains 11 and 15. Another possibility is that these metabolites have a high in/out flux as a result of rapid growth, and are at a lower steady-state concentration. This has been observed in flux analysis of fast-growing cultures. However, the lower concentrations of these metabolites in Strain 15 compared to Strain 11 did not correlate with a faster growth rate, only an increase in 23BD production (Fig. 4a,b). On the other hand, levels of the TCA cycle intermediates such as malate, fumarate and amino acids derived from either pyruvate or 2-oxoglutarate were elevated in Strains 3 (Fig. 4d and Supplementary Fig. 4). Malate is known as the main substrate for pyruvate biosynthesis in cyanobacteria. Since pyruvate is a key precursor to 23BD biosynthesis, a 15-fold increase in malate in malate is consistent with the remarkably enhanced 23BD production observed in Strain 15.

Effects of metabolic rewiring on 23BD production in darkness. When glucose is metabolized via the OPP pathway, NADPH production is balanced by oxidization steps in 23BD biosynthesis, regardless of whether carbon is metabolized by CO₂ fixation or the lower EMP pathway (Fig. 1c). This makes coupling the OPP and CO₂ fixation pathways desirable in terms of increasing NADPH availability for growth and chemical production, especially under periods of continuous darkness when NADPH is starkly limiting. Therefore, Strain 15 (galP-zwf-gnd + Δcp12:: prk-rbcLXS) should be able to generate sufficient reducing power (NADPH) to maintain 23BD production in the absence of light. To test this hypothesis, strains were tested for 23BD production in continuous dark conditions.

Strains 3 (galP), 11 (galP-zwf-gnd), 14 (Δcp12:: prk-rbcLXS) and 15 (11 + Δcp12:: prk-rbcLXS) were cultured with 20 mM NaHCO₃ and 10 g l⁻¹ glucose for 24 h in complete darkness. We expected that the combined overexpression of the OPP pathway and deregulation of GAPDH and PRK in Strain 15 would lead to more efficient 23BD production in dark conditions. R15P was elevated twofold in Strain 15 compared to Strain 14, while R15P was not detected in Strains 3 and 11 (Fig. 4f), indicating that deletion of cp12 allowed an increase in CB cycle carbon pools even in the dark. The biomass and 23BD production of Strain 15 were enhanced by 2.9- and 2.5-fold, respectively, compared to Strain 3, while Strain 11 only showed a 2.7-fold increase in biomass production (Fig. 4g). Also, although Strain 14 was able to fix carbon in darkness, as evidenced by the production of ¹³C-labelled 3PGA, its biomass and 23BD produced were lower than those of Strain 3 (Figs 3d and 4g). These data show that the cp12 deletion was effective in activating the CO₂ fixation pathway by deregulating PRK, and that the coupling with OPP overexpression redirected carbon flux to synergistically increase biomass and 23BD production.

To determine whether carbon fixation was also enhanced in Strains 3, 11, 14 or 15, cells were cultured with ¹³C-NaHCO₃ and unlabelled glucose for 24 h in continuous darkness, and ¹³C-labelled 23BD was quantified. A characteristic mass signal from ¹³C-NaHCO₃ was detected at 180 m/z, originating from either M + 2, M + 3 or M + 4 of derivatized 23BD produced by Strain 15, and this signal was not observed from Strains 3, 11 or 14 (Supplementary Fig. 7). This mass spectrum of 23BD produced by Strain 15 indicated that 14% of 23BD carbons came from CO₂. This result provides direct evidence that the CB cycle was active in Strain 15 even in darkness.

Figure 5 | Long-term production in continuous light and diurnal light conditions. Strain 15 (1 + galP-zwf-gnd + Δcp12:: prk-rbcLXS) was cultured in 10 ml BG11 media containing 15 g l⁻¹ glucose and 20 mM NaHCO₃ in continuous light conditions (a–c) and diurnal light conditions (d–f). Grey shades represent dark phases. Cells were induced with 1 mM IPTG on day 0. On days 3, 6 and 9, cells were collected by centrifugation and resuspended at an OD₇₃₀ of 5.0 in fresh production media. 23BD production (a,d), glucose consumption (b,e) and growth (c,f) profiles of Strain 15 where N = 3 biological replicates, and error bars represent s.d.
Long-term production of 23BD in continuous light conditions. For industrialization of photosynthetic chemical production, maximization of production titre is crucial since it greatly affects production recovery costs. To demonstrate that our engineering strategies yielded a strain able to produce high concentrations of 23BD over the long term, we cultured Strain 15 (gulP-zwF-gnd + Δcp12: prk-rbcLXS), the leading strain, with 15 g L⁻¹ glucose and 20 mM NaHCO₃ for 12 days under continuous illumination. To maximize production, we modified culture conditions (Supplementary Fig. 8), hypothesizing that increased isopropyl-β-D-thiogalactoside (IPTG) and A5 trace metal (Mn²⁺, Co²⁺, Cu²⁺ and MoO₄⁻²) concentrations would improve gene expression and cofactor availability. Cumulative 23BD production of Strain 15 was 12.6 g L⁻¹ on day 12, while total consumption of glucose was 18.5 g L⁻¹ (Fig. 5a–c). The yield was 136% of the TMY of 23BD calculated from glucose alone (0.5 g₂₃BD/gglucose). That the yield is above 100% indicates that both CO₂ and glucose were successfully utilized with at least 36% of carbons derived from CO₂. Given that the yield of 23BD in photomixotrophic conditions reported in our previous study was only 40% of the TMY, the efficiency of production achieved in this study represents a dramatic improvement.

Long-term production of 23BD in diurnal light conditions. To show that Strain 15 is also able to produce 23BD for an extended period of time without continuous illumination, we next cultured Strain 15 under diurnal light (12 h light/12 h dark) conditions with 15 g L⁻¹ glucose and 20 mM NaHCO₃ (Fig. 5d–f). Although optical density decreased during many of the dark phases (Fig. 5f), 23BD production lasted for 8 days, and was observed regardless of light availability, reaching a final titre of 5.7 g L⁻¹ (Fig. 5d). This production, and consumption of 6.1 g L⁻¹ glucose (Fig. 5e), corresponds to 195% of the TMY.

Discussion
Here we describe a strategy to improve CO₂ fixation and chemical production by rewiring carbon metabolism in cyanobacteria. We increased carbon flux from glucose by enhancing the OPP pathway, and then redirected it towards the carbon fixation step catalysed by RuBisCO. The engineered strain, Strain 15, showed a variety of advantages, including remarkable improvement of 23BD production and enhanced CO₂ fixation in both light and dark conditions.

Our integrative approach also explored improving phototrophic carbon fixation. We used glucose supplementation to boost R15P supply (Fig. 1b), and deleted cp12 to increase PRK-catalysed conversion of Ru5P into R15P. As expected, R15P content was the highest in Strain 15 grown with glucose in both light and dark conditions (Fig. 4e,f), suggesting that carbon flux in the CO₂ fixation pathway was significantly improved. These results also demonstrate the synergetic effects of deregulating carbon fixation and enhancing glucose catabolism for biomass and chemical production.

Improved carbon fixation in Strain 15 was also beneficial for long-term production under continuous and diurnal light conditions. With continuous lighting, we demonstrated a 23BD production titre of 12.6 g L⁻¹ from 18.5 g L⁻¹ glucose in 12 days. This yield (136%) greatly exceeded the TMY, indicating simultaneous use of glucose and CO₂. Unexpectedly, yield from diurnal light conditions was even more efficient: 5.7 g L⁻¹ 23BD produced from 6.1 g L⁻¹ glucose in 8 days, which is 195% of TMY. This suggests that CO₂ fixation and recycling were key to improving carbon yield, even during dark phases, and can be partially explained by changes in the carbon flux through the CB cycle during darkness (Supplementary Fig. 7). Furthermore, cell growth in diurnal conditions was decreased compared to constant light conditions (Fig. 5), suggesting that yield was improved by partitioning carbon to 23BD production rather than biomass formation.

An alternative strategy to photomixotrophic production has been previously demonstrated: a CO₂ fixation pathway consisting of PRK and RuBisCO was introduced into the heterotrophic organism, Saccharomyces cerevisiae, to improve ethanol yield. However, yield from galactose was only increased by 8% (0.44 gEthanol/gGalactose), and is still below the TMY (0.51 gEthanol/gGlucose). Succinate production from glycerol via carboxylation has been demonstrated to 133% of the TMY and the experimental carbon yield was 120% (ref. 41). However, carboxylation exclusive to a particular biosynthetic pathway is limited in applicability. By contrast, RuBisCO carboxylation is connected to central carbon metabolism, and thus to a range of chemical production pathways. Indeed, our method for improvement of carboxylation could be applied to increase photosynthetic production of a wide range of chemicals.

The strategy utilized in our study improved the productivity, titre, and yield of 23BD production (Fig. 5). Although cyanobacteria have been engineered to produce a number of compounds, the productivities and titres are much too low (most on the order of mg L⁻¹) to commercialize these technologies (Supplementary Fig. 9). In addition, strains developed for photoautotrophic production are restricted by light availability, even though dense cultures (OD730 > 5) are often required for efficient production. This presents a challenge since light penetration is hindered by mutual cell shading, resulting in decreased light availability per cell, and arrested cell growth and chemical production. In addition, in natural lighting conditions the active growth and production period falls to 8–14 h per day. Our approach overcomes these obstacles, providing significant improvements to chemical production in a variety of industrially relevant lighting and growth conditions. Economic analysis of 23BD production in diurnal light conditions using a semi-open pond illuminated by natural sunlight showed that operation profits could be increased fivefold in photomixotrophic conditions compared to photoautotrophic conditions (Supplementary Fig. 10 and Supplementary Note 2). This is despite the additional production costs required for photomixotrophic cultures, such as feedstock cost and steam sterilization of production media. In light conditions, it is advantageous to maximize cell density for chemical production. Sugar supplementation in these conditions may prove useful for overcoming density-dependent light deficiencies in phototrophic cultures. The described approach allows the engineered strains to produce and grow without light, capitalizing on night time hours. Glucose supplementation would also avoid issues with sunlight variability. In addition, the engineered strain’s high titre and productivity provide groundwork for production in smaller surface area bioreactors relative to outdoor photoautotrophic production. The recent development of less expensive light-emitting diode (LED) can provide optimal light intensity and wavelength for an energy-efficient production system. (Stored electricity generated from a renewable source, such as a solar panel or wind turbine, would be utilized to power the light-emitting diode.) Faster growth in a smaller surface area system may reduce the risk of contamination compared to current outdoor systems. Hence, this study demonstrates the many advantages of a next-generation scheme of cyanobacterial chemical production with potential for broad applicability in industrial production systems.

Methods
Reagents. The following reagents were obtained from Sigma-Aldrich: glucose, 1,3-propanediol, phenylboronic acid, cycloheximide, 23BD, acetoin, pyruvate, PEP,
and a diameter of 1.5 cm. Appropriate concentration (10 or 15 g l\(^{-1}\)) using the value of 0.22 g DCW l\(^{-1}\)

**Plasmid construction.** All primers and plasmids used in this study are listed in Supplementary Tables 1 and 2, respectively. The target genes and vector fragments used to construct plasmids were amplified using PCR with the primers and templates described in Supplementary Table 3. The resulting fragments were assembled by sequence and ligation-independent cloning.\(^{45}\)

**Culture conditions.** Unless otherwise specified, *S. elongatus* cells were cultured in BG11 medium with the addition of 50 mM NaHCO\(_3\). Cells were grown at 30°C with rotary shaking (100 r.p.m.) and light (30 \(\mu\)mol photons m\(^{-2}\) s\(^{-1}\) in the PAR range) provided by 66 cm 20 W fluorescent tubes. Light intensity was measured using a PAR quantum flux meter (Model MQ-200, Apogee Instruments). Dark conditions were achieved by wrapping tubes or flasks with aluminum foil and culturing them without light. Cell growth was monitored by measuring OD\(_{730}\) in a Microtek Synergy H1 plate reader (BioTek). All OD\(_{730}\) values were corrected for 1 cm path length. Cell biomass (dry cell weight (DCW)) was calculated from OD\(_{730}\) using the D-Glucose Assay Kit (Megazyme Inc.). The GC oven temperature was increased with a gradient of 3°C min\(^{-1}\) in the PAR

**Strain construction.** Strains used in this study are listed in Table 1. For transformation of *S. elongatus*\(^{43}\), cells at OD\(_{730}\) = 0.4 was collected from 2 ml of culture by centrifugation, washed and concentrated in 100 μl of fresh BG11 medium. Plasmid DNA (5 μg) was added to the cells. The tube was incubated overnight at 30°C. Cells were plated on a BG11 plate containing appropriate antibiotics and incubated at 30°C under constant light until colonies appear. A schematic of gene integration is summarized in Supplementary Fig. 2. Complete chromosomal segregation for the introduced fragments was achieved through propagation of multiple generations on selective agar plates. Correct recombinants were confirmed by colony PCR and sequencing to verify integration of heterologous genes in the targeted locus and complete removal of the gene targeted for deletion from the chromosomal DNA.

**Quantification of intracellular metabolites.** To prepare samples for metabolomics analysis, cultured cells were collected by vacuum filtration using a nylon membrane filter (0.45 μm, 47 mm, Whatman). Each filter was transferred to 15 ml centrifuge tube, and then immediately frozen in liquid nitrogen and stored at -80°C until analysis. Metabolite extraction, derivatization and analysis by GC-time of flight–MS was carried out by the West Coast Metabolomics Center at University of California, Davis. Metabolites were identified from MS spectra using the Bin2Base algorithm\(^{20,26}\). For determination of mass isotopomer distribution of aldehydes and 3-phosphoglycerate, the measured mass spectrum data of the unlabelled material were used to represent any and all combinations of isotopomer variations.\(^{46}\)

**Quantification of extracellular metabolites.** Glucose concentration in culture supernatants was determined by measuring the absorbance at 535 nm against a standard curve using pure acetoin. The GC oven temperature was set at 70°C for 3 min, and then increased with a gradient of 45°C min\(^{-1}\) until 300°C. The temperature of the injector was 225°C. The temperature was set at 200°C. For determination of mass isotopomer distribution, the measured mass spectrum data of the unlabelled 23BD was used to represent any and all combinations of isotopomer variations.\(^{46}\)

**Enzyme assays.** To determine the activities of the following enzymes: G6P dehydrogenase (ZWF), G6P dehydrogenase (GND), phosphoglucomutase isomerase (PGI), PRK and Rubisco, cells were grown with 10 g l\(^{-1}\) glucose, 200 mM NaHCO\(_3\), 0.1 mM IPTG, 10 mg l\(^{-1}\) thiamine for 24 h. Cells were collected, resuspended in 50 mM Tris-HCl buffer (pH 8.0) and the pH was adjusted to 7.0 with 0.1 M NaOH. Cells and 50 μM Mg\(_{2}\)O were added to a reaction mixture containing 50 mM Tris-HCl buffer (pH 8.0), 10 mM NaHCO\(_3\), 1 mM EDTA, 5 μl ATP, 0.3 mM NADH, 0.1 mM NADPH, 0.1 U G6PDH and 5 U G6PDH. A volume of 150 μl of 23BD was used to determine the activity of acetolactate synthase, and 5 U Rubisco. Reaction was performed at 30°C for 15 min, and the absorbance in the absence of NADPH at 340 nm was monitored to determine R15P content against a standard curve using pure R15P.
modifications. The substrate was replaced with 2-acetolactate freshly prepared from ethyl 2-acetoxy-2-methylacetocetate. To prepare 2-acetolactate, 50 μl of ethyl-2-acetoxy-2-methylacetocetate was mixed with 990 μl of water and 260 μl of 2 M NaOH was gradually added. The acidification step was omitted, and reactions were quenched by the transfer of 20 μl of reaction to wells in a 96-well plate each containing 80 μl 2.5 M NaOH.

Quantification of green fluorescent protein fluorescence. In prior to measurements of green fluorescent protein fluorescence, cultured S. elongatus cells were collected and resuspended in the equal volume of fresh BG11 medium. For fluorescence measurements, 488 nm was used for excitation, and emission was measured at 530 nm using a Microtect Synergy HT plate reader (BioTek).

Data availability. The authors declare that all data supporting the findings of this study are available within the article, its Supplementary Information file and from the corresponding author upon reasonable request.

References
1. Rasala, B. A. & Mayfield, S. P. Photosynthetic biomaterializing in green algae: production of recombinant proteins for industrial, nutritional, and medical uses. Photosynth. Res. 123, 227–239 (2015).
2. Machado, I. M. & Atsumi, S. Cyanobacterial biofuel production. J. Biotechnol. 162, 50–56 (2012).
3. Golden, S. S. & Canales, S. R. Cyanobacterial circadian clocks—timing is everything. Nat. Rev. Microbiol. 1, 191–199 (2003).
4. Ort, D. R. & Melis, A. Optimizing antenna size to maximize photosynthetic efficiency. Plant Physiol. 155, 79–85 (2011).
5. Heidorn, T. et al. Synthetic biology in cyanobacteria engineering and analyzing novel functions. Methods Enzymol. 497, 539–579 (2011).
6. Case, A. E. & Atsumi, S. Cyanobacterial chemical production. J. Biotechnol. 213, 101–116 (2016).
7. AngERMayer, S. A., Gorcs Rovira, A. & Hellingwerf, K. J. Metabolic engineering of cyanobacteria for the synthesis of commodity products. Trends Biotechnol. 33, 352–363 (2015).
8. Oliver, J. W. & Atsumi, S. Metabolic design for cyanobacterial chemical synthesis. Photosynth. Res. 120, 249–261 (2014).
9. Berla, B. M. et al. Synthetic biology of cyanobacteria: unique challenges and opportunities. Front. Microbiol. 4, 246 (2013).
10. Nouzi, N. E., Oliver, J. W. K. & Atsumi, S. Cyanobacteria as a platform for biofuel production. Front. Bioeng. Biotechnol. 1, 7 (2013).
11. McEwen, J. T., Machado, I. M., Connor, M. R. & Atsumi, S. Engineering Synechococcus elongatus PCC 7942 for continuous growth under diurnal conditions. Appl. Environ. Microbiol. 79, 1668–1675 (2013).
12. Oliver, J. W., Machado, I. M., Yoneda, H. & Atsumi, S. Cyanobacterial carbon dioxide fixation to 2,3-butanediol. Proc. Natl Acad. Sci. USA 110, 1249–1254 (2013).
13. McEwen, J. T., Kanno, M. & Atsumi, S. 2,3 Butanediol production in an obligate photoautotrophic and photomixotrophic cultures of Synechocystis sp. PCC 6803 examined by capillary electrophoresis electrospray ionization mass spectrometry. J. Exp. Bot. 59, 3009–3018 (2008).
14. Broedel, S. E. Jr & Wolf, R. E. Jr Genetic tagging, cloning, and DNA sequence of the Synechococcus sp. strain PCC 7942 gene (mgl) encoding 6-phosphogluconate dehydrogenase. J. Bacteriol. 172, 4023–4031 (1990).
15. Rubin, B. E. et al. The essential gene set of a photosynthetic organism. Proc. Natl Acad. Sci. USA 112, 19162–19167 (2015).
16. Raines, C. A. Increasing photosynthetic carbon assimilation in C3 plants to increase carbon productivity. Plant Physiol. 155, 1249–1254 (2011).
17. Tcherkez, G. G., Farquhar, G. D. & Andrews, T. J. Despite slow catalysis and enzyme interactions, biological systems can outperform non-biological counterparts. Nat. Biotechnol. 19, 6634–6639 (2013).
18. Savir, Y., Noor, E., Milo, R. & Tlusty, T. Cross-species analysis traces adaptation of Rubisco toward optimality in a low-dimensional landscape. Proc. Natl Acad. Sci. USA 107, 3475–3480 (2010).
19. Miyagawa, Y., TamoI, M. & Shigeoka, S. Overexpression of a cyanobacterial fructose-1,6-/sedoheptulose-1,7-biphosphatase in tobacco enhances photosynthesis and growth. Nat. Biotechnol. 19, 963–969 (2001).
20. Marcus, Y., Altman-Gurka, H., Wolff, Y. & Gurevitz, M. Rubisco mutagenesis provides new insight into limitations on photosynthesis and growth in Synechococcus PCC6803. J. Exp. Bot. 62, 4173–4182 (2011).
21. Li, M. Z. & Elledge, S. J. Harnessing homologous recombination in vitro to generate recombinant DNA via SLIC. Nat. Methods 4, 251–256 (2007).
22. Golden, S. S., Brusslan, J. & Haselkorn, R. Genetic engineering of the cyanobacterial chromosome. Methods Enzymol. 155, 213–231 (1987).
23. Oliver, J. W. & Atsumi, S. A carbon sink pathway increases carbon productivity in cyanobacteria. Metab. Eng. 29, 106–112 (2015).
24. Porter, W. H. & Auansukal, A. Gas-chromatographic determination of ethylene glycol in serum. Clin. Chem. 28, 75–78 (1982).
25. Jennings, M. E. & Matthews, D. E. Determination of complex isotopomer distribution from 13C-labeled glucose. Metab. Eng. 4, 202–216 (2002).
26. McEwen, J. T., Kanno, M. & Atsumi, S. 2,3 Butanediol production in an obligate photoautotrophic and photomixotrophic cultures of Synechocystis sp. strain PCC 7942 gene (mgl) encoding 6-phosphogluconate dehydrogenase. J. Bacteriol. 172, 4023–4031 (1990).
27. Rubin, B. E. et al. The essential gene set of a photosynthetic organism. Proc. Natl Acad. Sci. USA 112, 19162–19167 (2015).
28. Raines, C. A. Increasing photosynthetic carbon assimilation in C3 plants to increase carbon productivity. Plant Physiol. 155, 1249–1254 (2011).
29. Tcherkez, G. G., Farquhar, G. D. & Andrews, T. J. Despite slow catalysis and enzyme interactions, biological systems can outperform non-biological counterparts. Nat. Biotechnol. 19, 6634–6639 (2013).
30. Savir, Y., Noor, E., Milo, R. & Tlusty, T. Cross-species analysis traces adaptation of Rubisco toward optimality in a low-dimensional landscape. Proc. Natl Acad. Sci. USA 107, 3475–3480 (2010).
31. Miyagawa, Y., TamoI, M. & Shigeoka, S. Overexpression of a cyanobacterial fructose-1,6-/sedoheptulose-1,7-biphosphatase in tobacco enhances photosynthesis and growth. Nat. Biotechnol. 19, 963–969 (2001).
32. Marcus, Y., Altman-Gurka, H., Wolff, Y. & Gurevitz, M. Rubisco mutagenesis provides new insight into limitations on photosynthesis and growth in Synechococcus PCC6803. J. Exp. Bot. 62, 4173–4182 (2011).
33. Li, M. Z. & Elledge, S. J. Harnessing homologous recombination in vitro to generate recombinant DNA via SLIC. Nat. Methods 4, 251–256 (2007).
34. Golden, S. S., Brusslan, J. & Haselkorn, R. Genetic engineering of the cyanobacterial chromosome. Methods Enzymol. 155, 213–231 (1987).
35. Oliver, J. W. & Atsumi, S. A carbon sink pathway increases carbon productivity in cyanobacteria. Metab. Eng. 29, 106–112 (2015).
36. Porter, W. H. & Auansukal, A. Gas-chromatographic determination of ethylene glycol in serum. Clin. Chem. 28, 75–78 (1982).
37. Jennings, M. E. & Matthews, D. E. Determination of complex isotopomer patterns in isotopically labeled compounds by mass spectrometry. Anal. Chem. 77, 6435–6444 (2005).
38. Nozni, E. & Atsumi, S. Genome engineering of the 2,3-butanediol biosynthetic pathway for tight regulation in cyanobacteria. ACS Synth. Biol. 4, 1197–1204 (2015).

Acknowledgements
This work was supported by the Asahi Kasei Corporation and the National Science Foundation (CBET-1132442 and CBET-1349663). A.L.C. is supported by an NSF Graduate Research Fellowship. We thank Nicole E. Nozu, Anna E. Case and Christine A. Raboinovich-Deere for critical reading of the manuscript.

Author contributions
M.K., A.L.C. and S.A. analysed data; M.K., A.L.C. and S.A. wrote the manuscript.
Additional information
Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: The authors declare no competing financial interests.

Reprints and permission information is available online at http://npg.nature.com/reprintsandpermissions/

How to cite this article: Kanno, M. et al. Global metabolic rewiring for improved CO₂ fixation and chemical production in cyanobacteria. Nat. Commun. 8, 14724 doi: 10.1038/ncomms14724 (2017).

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/

© The Author(s) 2017