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In Vitro and In Vivo Supramolecular Modification of Biomembranes Using a Lipidated Coiled-Coil Motif**

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The molecular building blocks available in biological systems self-assemble into defined structures in an extremely controlled manner. These structures must be flexible and adaptive to the environment in order to carry out their function in a regulated manner. Therefore, nature uses multiple weak interactions (e.g. hydrogen bonding and van der Waals interactions) to act as the glue to hold these structures together. When many weak interactions cooperatively combine, relatively stable entities are produced, which retain the ability to respond to external stimuli such as fluctuations in ion concentration, pH, and temperature.

For many years, nature has been a source of inspiration for supramolecular chemistry.^[1] Scientists typically follow the bottom-up approach and design relatively simple molecules which assemble into functional materials with well-defined properties.^[2] Recent progress has resulted in molecular systems that are responsive to multiple stimuli and are therefore highly controlled, emulating nature ever more closely. A relatively new development is the application of supramolecular constructs in *in vitro* and *in vivo* environments to directly study and influence biological processes in live cells. Chemically tailored systems can be integrated into cell membranes, for example. This enables the modification or regulation of cellular behavior through external artificial signals.^[3] There are two approaches for introducing chemical species into a cell membrane by supramolecular chemistry: 1) specific binding of guest molecules to membrane-anchored biomolecules such as native proteins^[4] and 2) nonspecific labeling of membranes with the aid of hydrophobic and electrostatic interactions or through a chemical crosslinker.^[5]

Lipidated peptides are particularly good candidates for application in biological systems as their aggregation behavior can be controlled by carefully balancing the hydro-

phobicity of the anchor and the hydrophilicity of the cargo;^[6] this aids the incorporation of lipidated peptides into membranes. Here we describe the use of a coiled-coil motif as the peptide segment, a highly specific recognition system that can be introduced into live cells. The coiled-coil motif acts as molecular Velcro and can thus be used to link distinct molecular constructs.^[7] An example of the specific labeling of proteins through coiled-coil formation was recently supplied by Matsuzaki et al.^[8] Surface modification through the non-specific binding of polymers to cell membranes has also been studied, for example by Ijiro et al.^[9] Lipid-grafted polymers adhere to cell membranes and could potentially act as a scaffold to which a wide range of functional moieties could be attached, thereby intervening in the chemistry of the cell. Furthermore, cationic graft copolymers have also been shown to interact electrostatically with cell membranes, resulting in chemically altered cell membranes.^[10] Although these examples illustrate that *in vitro* membrane functionalization is a highly rewarding strategy, there are currently no examples of efficient *in vivo* strategies. Therefore, it is our goal to transiently modify lipid membranes through specific supramolecular interactions in *in vitro* and *in vivo* environments. For this purpose, we use a pair of complementary coiled-coil-forming lipidated peptides (E and K peptides)^[7a,11] to specifically introduce a noncovalent and bio-orthogonal recognition motif to biological membranes (Scheme 1).

Here, we describe a generic supramolecular tool which allows us to rapidly and efficiently form coiled-coil motifs at the surface of biological membranes. This is of interest as a wide range of molecular constructs can be introduced to the surface of the cell in this way. Coiled-coil-forming peptides E [(EIAALEK)₃] and K [(KIAALKE)₃]^[12] were first covalently conjugated to PEG₁₂ spacers (PEG = polyethylene glycol). Subsequently, a cholesterol moiety was coupled to the pegylated peptides yielding CPE and CPK (Scheme 1A).^[13] The cholesterol moiety allows for the immediate insertion of the lipidated peptides into membranes through hydrophobic interactions and the PEG₁₂ moiety was incorporated to aid efficient molecular recognition between the peptide segments E and K. Recently we showed that upon the addition of micellar solutions of either CPE or CPK to plain liposomes, the lipidated peptides spontaneously inserted into liposomal membranes.^[13]

In the current study, CHO cell membranes (CHO = Chinese hamster ovary) and the skin of zebrafish embryos were modified with coiled-coil-forming peptides by the addition of a micellar solution of CPE or CPK, resulting in immediate incorporation of these amphiphiles into the membranes. Subsequently, the complementary peptide was added, result-

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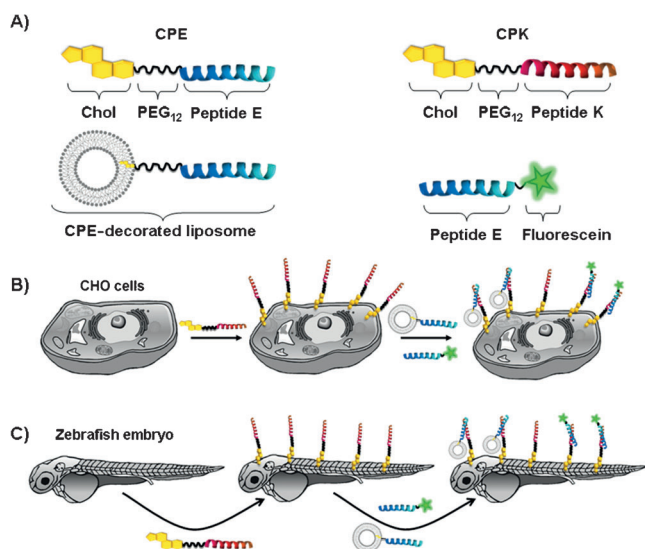
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Scheme 1. A) The lipidated E and K peptides, a CPE-decorated liposome, and the fluorescently labeled E peptide. B) Anchoring of CPK into the CHO cell membrane and subsequent coiled-coil formation with fluorescently labeled peptide E or CPE-decorated liposomes. C) Anchoring of CPK into the skin of a zebrafish embryo and subsequent coiled-coil formation with fluorescently labeled peptide E or CPE-decorated liposomes. (Images are not to scale.)

ing in coiled-coil formation. This system thus enables the docking of a wide variety of molecular constructs through coiled-coil formation (Scheme 1 B,C).

In order to establish coiled-coil formation at the surfaces of CHO cells, we first confirmed that the cholesterol anchor inserted into the membranes of the cells. Carboxyfluorescein-labeled CPE was added at a final concentration of $5\ \mu\text{M}$, which is the standard concentration used for the addition of lipidated peptides in this study. After incubation for a few minutes, confocal microscopy was used to image the treated cells. As expected, the cells showed highly fluorescent membranes, indicating that CPE was efficiently incorporated into the cell membrane (Figure 1). Surprisingly, some internalization of CPE was observed. A similar effect was observed by Irvine et al., who used a diacyllipid (C_{18}) and

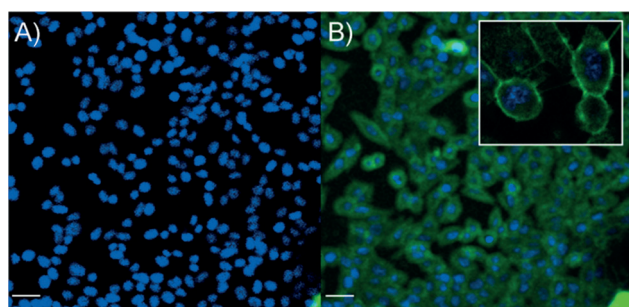


Figure 1. Confocal microscopy images of CHO cells. The cells were treated with the nuclear stain Hoechst for 30 min and then incubated with fluorescently labeled CPE at 37°C . Images were obtained A) 0 min and B) 10 min after the addition of fluorescent CPE ($5\ \mu\text{M}$ final concentration). The inset in (B) shows a magnified image, revealing some internalization of the lipopeptide. Scale bar: $25\ \mu\text{m}$.

cholesterol to anchor oligonucleotides into cell membranes.^[14] These lipidated oligonucleotides anchored on the cell membrane in 30 min, whereas lipidated peptides could anchor in less than 5 min. One explanation for this is that as oligonucleotides are more anionic than the peptides used in this study, electrostatic repulsion might delay incorporation into the membrane. It was also found that incorporating a PEG₂₄ spacer between the oligonucleotide and lipid segments drastically reduced the insertion capability; a PEG₁₂ spacer did not hinder membrane insertion.

Subsequently, we investigated whether the peptides at the CHO cell surface were accessible and capable of forming coiled coils with the complementary peptide. For this, the cells were treated with a CPK solution for 5 min and then washed with HBSS buffer (HBSS = Hank's balanced salt solution) to remove any unbound CPK. Next, a solution of carboxyfluorescein-labeled peptide E (final concentration $2.5\ \mu\text{M}$) was added to the cells and incubated for 10 min. The cells were washed three more times to remove any unbound peptide E. Confocal imaging of the CHO cells revealed that the fluorescent signal was located at the cell membranes (Figure 2). In a control experiment the addition of CPK was

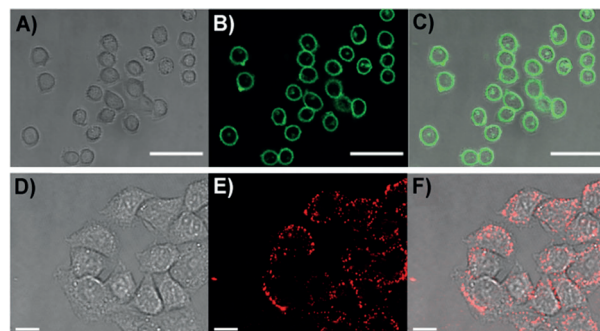


Figure 2. A–C) Confocal microscopy images (A: bright field, B: fluorescence, C: overlay) indicating coiled-coil formation between peptides E and K at the surface of CHO cells. First, CPK in HBSS ($5\ \mu\text{M}$ final concentration) was added to the cells which were incubated for 5 min. Subsequently, fluorescently labeled peptide E was added ($2.5\ \mu\text{M}$ final concentration) and the cells were incubated for 5 min. The cells were washed three times with HBSS before imaging. All experiments (incubations) were performed at 37°C . Scale bar: $50\ \mu\text{m}$. D–F) Confocal microscopy images (D: bright field, E: fluorescence, F: overlay) indicating docking of liposomes at the surface of CHO cells. First, CPK in HBSS ($5\ \mu\text{M}$ final concentration) was added to the cells which were incubated for 5 min. Subsequently, CPE-decorated liposomes ($0.25\ \text{mM}$ total lipid concentration and 1 mol% CPE) with 1 mol% DHPE-TR were added to the cells. All the experiments were performed at 37°C . Scale bar: $25\ \mu\text{m}$.

omitted and as a result the cells showed no fluorescence (Supporting Information, Figure S1). These observations strongly suggest efficient coiled-coil formation between the membrane-bound peptide E and peptide K. Additionally, the opposite route could also be taken, in other words, first addition of CPE to the cells, followed by treatment with the fluorescent K peptide; this also yielded specific coiled-coil formation (Supporting Information, Figure S2). Also, in both experiments some internalization of the surface-bound fluo-

rescence was observed. These results show that both CPK and CPE can be used to functionalize the CHO cell membrane.

In contrast, the protein-engineering approach described by Matsuzaki et al. only led to coiled-coil formation when the engineered membrane protein contained peptide E, but not the other way around.^[15] Furthermore, our system potentially allows for the modification of cell membranes of various cell lines without the use of laborious protein engineering protocols. Additionally, our supramolecular method is fast and the necessary peptides can be synthesized within one day, as opposed to the protein engineering approach, where the modified receptor could take significantly more time.

These initial studies demonstrated that we can form coiled coils at the surface of CHO cells by simply incubating a cholesterol peptide with the CHO cells and subsequently adding the complementary peptide. This system can be used to dock various molecules and nanoparticles to the cells. We have recently shown that 100-nm liposomes can be immobilized at interfaces mediated by coiled-coil formation between peptides E and K.^[16] Therefore it was of interest to study whether liposome docking is also possible at a biological interface. Instead of adding a fluorescent peptide to the pretreated cells, we incorporated the complementary peptide into liposomes (Figure 2). After membrane functionalization with CPK, fluorescent liposomes, containing 1 mol% DHPE-TR (Texas Red 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine) and 1 mol% CPE were incubated with the cells for 5–10 min. The cells were washed three times before confocal images of the cells were obtained (Figure 2). The images show increased fluorescence at the cell membranes with some internalization. Furthermore, with the CPE liposomes by themselves, that is, without the presence of peptide K at the cell membranes, no docking to the cells was observed (Supporting Information, Figure S3). This shows that large supramolecular assemblies can be docked onto membranes in an *in vitro* environment, using the orthogonal noncovalent coiled-coil motif. These findings enable future applications such as biophysical studies and pharmaceutical research. For example, adjuvant modified cells can be injected inside animals, which might lead to cell-based therapy applications.^[17]

Preclinical studies are a required step in translating innovations from a laboratory setting to the clinic. Recent restrictions on animal testing have increased the demand for alternative animal models. Therefore, zebrafish have become an attractive model organism in recent years as they bridge the gap between cell-based assays and mammalian models.^[18] Due to the low cost of husbandry, high reproductive capability, optical transparency of the embryos, and the ease of experimental manipulation, zebrafish might be a valuable tool in the development of novel drug-delivery systems for human disease therapy.^[19] Consequently, we studied whether our coiled-coil-based system could also function in zebrafish embryos. To the best of our knowledge, the surface modification of zebrafish has not been studied before (Scheme 1C).

One-day-old zebrafish embryos were pretreated with CPK in egg water. Next, fluorescent-labeled peptide E was added and incubated with the zebrafish embryos for 30 min, which were then washed three times with egg water prior to

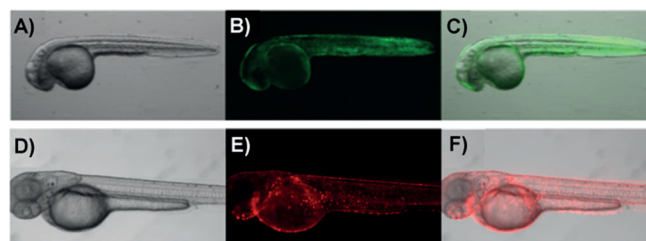


Figure 3. A–C) Coiled-coil formation between CPK and fluorescently labeled peptide E on the skin of one-day-old zebrafish embryos. The embryos were pretreated with CPK in egg water (5 μM final concentration) and incubated with complementary fluorescent peptide E (2.5 μM final concentration) for 30 min at 34 $^{\circ}\text{C}$ (A: bright field, B: fluorescence, C: overlay). D–F) Docking of liposomes on the skin of zebrafish embryos through coiled-coil formation. Fluorescent microscope images of two-day-old zebrafish embryos pretreated with CPK in egg water (5 μM final concentration) and incubated with 0.25 mM liposomes (total lipid concentration) bearing 1 mol% CPE and 1 mol% DHPE-TR on the membrane for 30 min at 34 $^{\circ}\text{C}$ (D: bright field, E: fluorescence, F: overlay). Embryos are shown in lateral view, anterior to the left.

imaging. Fluorescence imaging revealed that the skin of the embryos was highly fluorescent (Figure 3A–C). This is a strong indication that we accomplished efficient coiled-coil formation *in vivo*. Furthermore, the control experiment in which the embryos were not pretreated with CPK showed no significant fluorescence (Supporting Information, Figure S4). This strongly indicates that CPK efficiently decorated the skin of the zebrafish and that the peptide was available for coiled-coil formation with its complementary peptide.

Interestingly, we observed functionalization of the whole embryo. This is presumably because the epidermis of zebrafish embryos consist only of living cells, unlike terrestrial vertebrates in which dead, keratinized cells are present.^[20] Similar to *in vitro* experiments, functionalizing zebrafish embryos with CPE followed by incubation with fluorescently labeled K peptide was also attempted. Surprisingly, this alternative route did not result in efficient coiled-coil formation on the skin of the zebrafish embryos, which is in contrast to the cell-based study. Fish are known to have a different lipid composition than terrestrial vertebrates, as they contain high levels of polyunsaturated fatty acids.^[21] Presumably, this lipid composition favors the positively charged K peptide over the negatively charged E peptide. However, further studies are necessary to elucidate the difference in the behavior of the peptides at the surface of CHO cells and zebrafish embryos.

As a final investigation, we examined whether docking of liposomes, as previously demonstrated for the CHO cells, could also be achieved *in vivo* on the skin of two-day-old zebrafish embryos. In each group, embryos were selected and first incubated with CPK in egg water for 30 min at 34 $^{\circ}\text{C}$. After 30 min embryos were washed and then incubated with 1 mol% CPE-decorated fluorescently labeled liposomes for 30 min at 34 $^{\circ}\text{C}$. Microscopic analysis showed that the skin lit up brightly, revealing the docking of liposomes to the skin of the embryos (Figure 3D–F). Control experiments in which no CPK was administered displayed no significant fluorescence,

showing that liposome docking can be attributed to the specific coiled-coil interactions between peptides E and K (Supporting Information, Figure S5).

As zebrafish have been intensively studied as a model for various infectious diseases,^[22] this study enables future studies in which infected zebrafish embryos are treated using therapeutic liposomes controlled by supramolecular coiled-coil interactions. Since liposomes contain both hydrophilic and hydrophobic regions, there are a wide range of possibilities for encapsulating drugs for controlled release into the zebrafish embryos. Therefore, in future studies we will screen a variety of therapeutic substances to investigate their effect on a range of diseases.^[23] Further, it is possible to introduce in vivo selectivity in our model system. For example, aptamers could be used to specifically target a specific cell type or an organ, either by covalently conjugating the aptamer to the lipidated peptide, or by using mixed micelles (CPK + lipidated aptamer). A technological alternative would be the use of automated microinjection systems to inject the lipidated peptide or mixed micelles locally into zebrafish embryos or in a main blood vessel,^[24] allowing for specificity towards an organ or cell type.

In conclusion, we have designed a generic tool to modify the surface of live cells and zebrafish embryos in a fast and efficient manner. By using a supramolecular approach, employing an orthogonal coiled-coil-forming peptide pair, it was possible to functionalize the membrane of CHO cells and the skin of zebrafish embryos. This method can be used to dock a wide range of materials at a high local concentration to CHO cells and zebrafish embryos, which was demonstrated by liposome docking in vitro and in vivo. This generic method holds much promise as an elegant and fast tool in supramolecular functionalization of biomembranes and drug delivery. It also enables biophysical studies of biological processes occurring at membranes.

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- [1] a) J. Voskuhl, B. J. Ravoo, *Chem. Soc. Rev.* **2009**, *38*, 495–505; b) D. A. Uhlenheuer, D. Wasserberg, C. Haase, H. D. Nguyen, J. H. Schenkel, J. Huskens, B. J. Ravoo, P. Jonkheijm, L. Brunsveld, *Chem. Eur. J.* **2012**, *18*, 6788–6794; c) R. V. Ulijn, A. M. Smith, *Chem. Soc. Rev.* **2008**, *37*, 664–675; d) M. A. Kostianen, O. Kasyutich, J. Cornelissen, R. J. M. Nolte, *Nat. Chem.* **2010**, *2*, 394–399.
- [2] a) J. M. Lehn, *Angew. Chem.* **1990**, *102*, 1347–1362; *Angew. Chem. Int. Ed. Engl.* **1990**, *29*, 1304–1319; b) C. A. Mirkin, R. L. Letsinger, R. C. Mucic, J. J. Storhoff, *Nature* **1996**, *382*, 607–609; c) L. C. Palmer, S. I. Stupp, *Acc. Chem. Res.* **2008**, *41*, 1674–1684; d) R. P. Sijbesma, E. W. Meijer, *Curr. Opin. Colloid Interface Sci.* **1999**, *4*, 24–32.
- [3] B. R. Peterson, *Org. Biomol. Chem.* **2005**, *3*, 3607–3612.
- [4] E. M. Sletten, C. R. Bertozzi, *Angew. Chem.* **2009**, *121*, 7108–7133; *Angew. Chem. Int. Ed.* **2009**, *48*, 6974–6998.
- [5] a) J. H. Felgner, R. Kumar, C. N. Sridhar, C. J. Wheeler, Y. J. Tsai, R. Border, P. Ramsey, M. Martin, P. L. Felgner, *J. Biol. Chem.* **1994**, *269*, 2550–2561; b) F. Borcard, A. Godinat, D. Staedler, H. C. Blanco, A. L. Dumont, C. Chapuis-Bernasconi, C. Scaletta, L. A. Applegate, F. K. Juillerat, U. T. Gonzenbach, S. Gerber-Lemaire, L. Juillerat-Jeanerret, *Bioconjugate Chem.* **2011**, *22*, 1422–1432; c) D. Dutta, A. Pulsipher, W. Luo, H. Mak, M. N. Yousaf, *Bioconjugate Chem.* **2011**, *22*, 2423–2433.
- [6] F. Versluis, H. R. Marsden, A. Kros, *Chem. Soc. Rev.* **2010**, *39*, 3434–3444.
- [7] a) A. L. Boyle, D. N. Woolfson, *Chem. Soc. Rev.* **2011**, *40*, 4295–4306; b) H. Gradišar, S. Božič, T. Doles, D. Vengust, I. Hafner-Bratkovič, A. Mertelj, B. Webb, A. Šali, S. Klavžar, R. Jerala, *Nat. Chem. Biol.* **2013**, *9*, 362–366; c) B. Apostolovic, M. Danial, H. A. Klok, *Chem. Soc. Rev.* **2010**, *39*, 3541–3575; d) H. R. Marsden, A. V. Korobko, E. N. M. van Leeuwen, E. M. Pouget, S. J. Veen, N. Sommerdijk, A. Kros, *J. Am. Chem. Soc.* **2008**, *130*, 9386–9393; e) J. M. Mason, K. M. Arndt, *ChemBioChem* **2004**, *5*, 170–176; f) W. A. Petka, J. L. Harden, K. P. McGrath, D. Wirtz, D. A. Tirrell, *Science* **1998**, *281*, 389–392.
- [8] Y. Yano, K. Matsuzaki, *Biochim. Biophys. Acta Biomembr.* **2009**, *1788*, 2124–2131.
- [9] R. Kamitani, K. Niikura, T. Okajima, Y. Matsuo, K. Ijiro, *ChemBioChem* **2009**, *10*, 230–233.
- [10] J. T. Wilson, V. R. Krishnamurthy, W. X. Cui, Z. Qu, E. L. Chaikof, *J. Am. Chem. Soc.* **2009**, *131*, 18228–18229.
- [11] a) H. Robson Marsden, A. V. Korobko, T. Zheng, J. Voskuhl, A. Kros, *J. Biomater. Sci. Polym. Ed.* **2013**, *1*, 1046–1054; b) T. Zheng, J. Voskuhl, F. Versluis, H. R. Zope, I. Tomatsu, H. R. Marsden, A. Kros, *Chem. Commun.* **2013**, *49*, 3649–3651.
- [12] a) J. R. Litowski, R. S. Hodges, *J. Biol. Chem.* **2002**, *277*, 37272–37279; b) H. Robson Marsden, N. A. Elbers, P. H. H. Bomans, N. Sommerdijk, A. Kros, *Angew. Chem.* **2009**, *121*, 2366–2369; *Angew. Chem. Int. Ed.* **2009**, *48*, 2330–2333; c) B. Apostolovic, S. P. E. Deacon, R. Duncan, H. A. Klok, *Macromol. Rapid Commun.* **2011**, *32*, 11–18; d) M. B. Hansen, W. P. R. Verduren, E. H. P. Leunissen, I. Minten, J. C. M. van Hest, R. Brock, D. Lowik, *ChemBioChem* **2011**, *12*, 2294–2297.
- [13] F. Versluis, J. Voskuhl, B. van Kolck, H. Zope, M. Bremmer, T. Albrechtse, A. Kros, *J. Am. Chem. Soc.* **2013**, *135*, 8057–8062.
- [14] H. P. Liu, B. Kwong, D. J. Irvine, *Angew. Chem.* **2011**, *123*, 7190–7193; *Angew. Chem. Int. Ed.* **2011**, *50*, 7052–7055.
- [15] Y. Yano, A. Yano, S. Oishi, Y. Sugimoto, G. Tsujimoto, N. Fujii, K. Matsuzaki, *ACS Chem. Biol.* **2008**, *3*, 341–345.
- [16] J. Voskuhl, C. Wendeln, F. Versluis, E. C. Fritz, O. Roling, H. Zope, C. Schulz, S. Rinnen, H. F. Arlinghaus, B. J. Ravoo, A. Kros, *Angew. Chem.* **2012**, *124*, 12786–12790; *Angew. Chem. Int. Ed.* **2012**, *51*, 12616–12620.
- [17] M. T. Stephan, J. J. Moon, S. H. Um, A. Bershteyn, D. J. Irvine, *Nat. Med.* **2010**, *16*, 1035–1135.
- [18] S. A. Brittijn, S. J. Duivesteyn, M. Belmamoune, L. F. M. Bertens, W. Bitter, J. D. De Bruijn, D. L. Champagne, E. Cuppen, G. Flik, C. M. Vandenbroucke-Grauls, R. A. J. Janssen, I. M. L. De Jong, E. R. De Kloet, A. Kros, A. H. Meijer, J. R. Metz, A. M. Van der Sar, M. J. M. Schaaf, S. Schulte-Merker, H. P. Spaink, P. P. Tak, F. J. Verbeek, M. J. Vervoordeldonk, F. J. Vonk, F. Witte, H. P. Yuan, M. K. Richardson, *Int. J. Dev. Biol.* **2009**, *53*, 835–850.
- [19] C. Chakraborty, C. H. Hsu, Z. H. Wen, C. S. Lin, G. Agoramoorthy, *Curr. Drug Metab.* **2009**, *10*, 116–124.
- [20] D. Le Guellec, G. Morvan-Dubois, J. Y. Sire, *Int. J. Dev. Biol.* **2004**, *48*, 217–231.
- [21] a) R. J. Henderson, D. R. Tocher, *Prog. Lipid Res.* **1987**, *26*, 281–347; b) M. Holtta-Vuori, V. T. V. Salo, L. Nyberg, C. Brackmann, A. Ejnder, P. Panula, E. Ikonen, *Biochem. J.* **2010**, *429*, 235–242.

- [22] a) A. M. van der Sar, B. J. Appelmelk, C. Vandenbroucke-Grauls, W. Bitter, *Trends Microbiol.* **2004**, *12*, 451–457; b) G. J. Lieschke, P. D. Currie, *Nat. Rev. Genet.* **2007**, *8*, 353–367.
- [23] R. Carvalho, J. de Sonnevile, O. W. Stockhammer, N. D. L. Savage, W. J. Veneman, T. H. M. Ottenhoff, R. P. Dirks, A. H. Meijer, H. P. Spaik, *PLoS One* **2011**, *6*, e16779.
- [24] W. J. Veneman, O. W. Stockhammer, L. de Boer, S. A. J. Zaat, A. H. Meijer, H. P. Spaik, *BMC Genomics*, **2013**, *14*, 255.
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