
Supplementary information

**Reverse and forward engineering of
Drosophila corneal nanocoatings**

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Supplementary Information for

Reverse and forward engineering of *Drosophila* corneal nanocoatings

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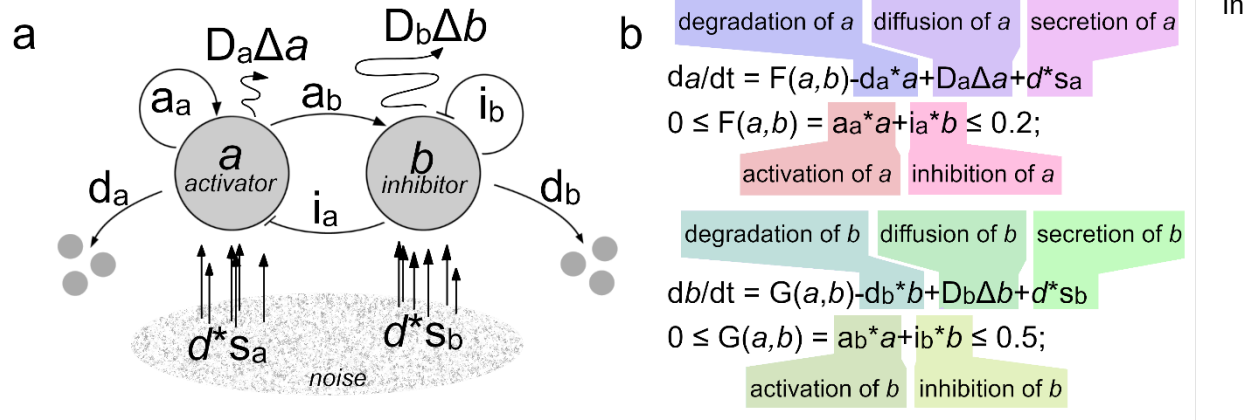
This Supplementary Information consists of Supplementary Notes 1-6 (including Supplementary Figures 1-17 and Supplementary Tables 1-3), Supplemental Methods and Supplementary References, altogether providing exhaustive description of details of the technical aspects of our work.

Supplementary Note 1. 3D Turing modelling

Below we provide details of the Turing model we have developed to gain insights into the potential physical mechanism underlying formation of insect corneal nanocoatings, and biological implications of this mechanism. We also compare the Turing model with an alternative block copolymer model, showing that the latter is inferior in recapitulating our experimental observations.

The Turing mechanism evokes antagonistic interactions of two morphogens – slower-diffusing Activator and faster-diffusing Inhibitor^{5,6}. Other details of the model are schematically represented in Supplementary Fig.1 below and in the standard Turing model imply, among other characteristics, that the Activator increases, and Inhibitor decreases, the concentrations (or productions) of themselves^{5,6}. In our adaptation of the Turing model to corneal nanocoatings, we presumed instead that “both types of molecules are produced and secreted into the lens by the cone cells in a preset manner, and it is the

ability of these molecules to aggregate/polymerize that is controlled by the Turing-type mechanisms” (cited from the p.2 of the Supplementary materials of ref⁴).

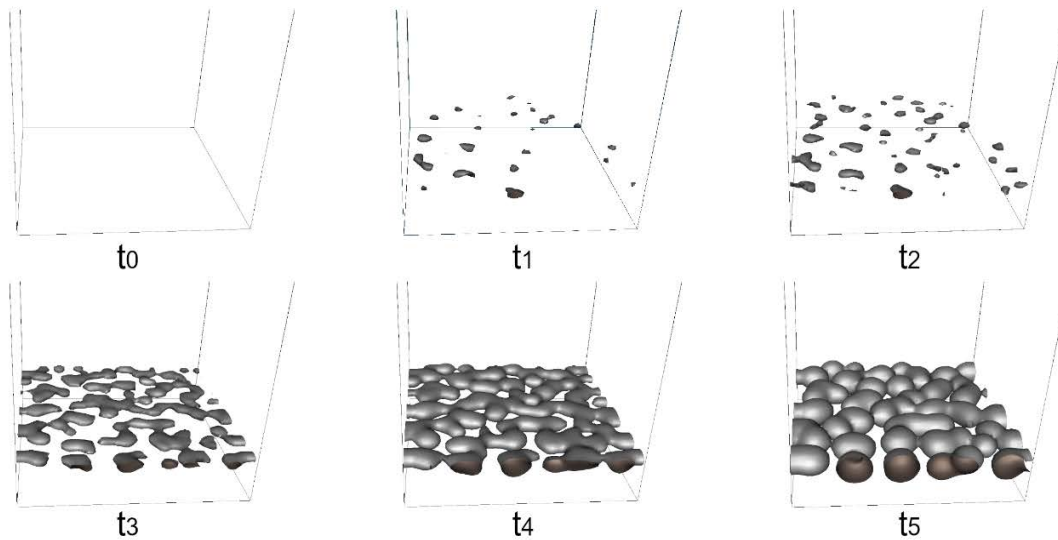


Supplementary Figure 1. 3D Turing modeling. Scheme and equations of the 3D Turing model developed in this study. At the bottom of (a), random secretion of the two Turing morphogens (s_a and s_b for the Activator (a), and the Inhibitor (b), respectively) is indicated; the production of the morphogens is associated with white noise (d). The Activator can degrade (d_a), self-activate (a_a) and activate the Inhibitor (a_b), while having a low diffusion rate ($D_a \Delta a$). The Inhibitor can also be degraded (d_b) or self-inhibited (i_b), or inhibit the Activator (i_a), and has a high diffusion rate ($D_b \Delta b$). In (b), these interactions are presented mathematically, in the form of equations and comments to them.

this work, we expanded the model to include local secretion of morphogenes in 3D space. We suppose that a key aspect of the interaction between the Activator (Retinin) and the Inhibitor (wax) is the induced-fit folding of Retinin (see Fig.3 and section “Induced folding of Retinin by waxes” of the main text). This interaction leads to formation of reversible complexes of the Activator and Inhibitor; these complexes can further assemble into nanoparticles. This process involves an increasing number of Retinin molecules (a_a , self-activation) and wax molecules (a_b , activation of the Inhibitor by Activator) to join the complex. Wax is also predicted, upon the complex reaching a particular size and/or structure, to restrict further incorporation of Retinin and wax molecules (i_a , inhibition of the Activator by Inhibitor; i_b , self-inhibition). Being unstable, the complex is in a state of dynamic equilibrium with the medium, forming a pool of reagents dissolved in it (d_a , d_b – degradation). From the “dissolved” state, Retinin and wax can be recruited again as part of the complex; the amount of the recruited material cannot be negative or higher than the amount of the surrounding material (bounds for $F(a,b)$ and $G(a,b)$).

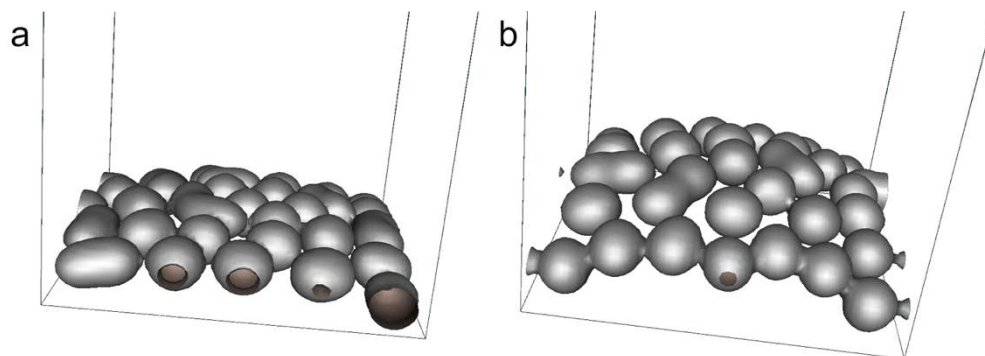
In this work, we made an effort to maximally approximate the model to the existing data about formation of corneal nanocoatings¹¹. Simulation is made in a 3D box (64x64x64px), with steps of 0.1. The forward Euler integration with the Laplacians computed applying a 27-point stencil⁴² was used. As a substitute for cone cells secreting the reagents, we used a ball with the radius equal to 6400px (placed at the bottom of the box so that only the upper border of the ball penetrates into the box, forming an almost flat surface at a distance of 3.5px from its lower edge), with a constant noise value (from 0.01 to 0.02) of the increment of reagents. Considering the diffusion and degradation of reagents, their concentration gradually decreases from the centre to the edge of the ball. A certain time after the start of the simulation, thin and

scattered high-density structures begin to appear above the boundary of the ball (the concentration of the reagents together inside the structures is above 1.5, with a maximal secretion level (d) of 0.02) (Supplementary Fig.2). It is important to note that in case of simulation that considers the curvature of the lens (Supplementary Fig.3a), or even exceeds it (Supplementary Fig.3b), the principle of formation of the nanostructures does not change, nor does the final type of the structures produced.



Supplementary Figure 2. Different timepoints of the nanopatterning simulated in 3D. The patterns obtained at different simulation time points resemble well the natural corneal nanocoatings forming at different time points (ref¹¹) and include transition from patches (time period t_1 and t_2) to mature nipple-like nanostructures (t_5).

This process is very similar to the dynamics of nanocoating formation observed in developing butterflies¹¹. In this latter case, the nanostructures also first appear in the form of thin scattered seals above the microvilli and upon gradual expansion form a stereotyped 3D pattern. It is important to stress that the corneal lens forms under this layer of nanostructures and later than the nanostructures appear¹¹. We depict the lens material in the form of a cross-section passing through the 3D pattern.

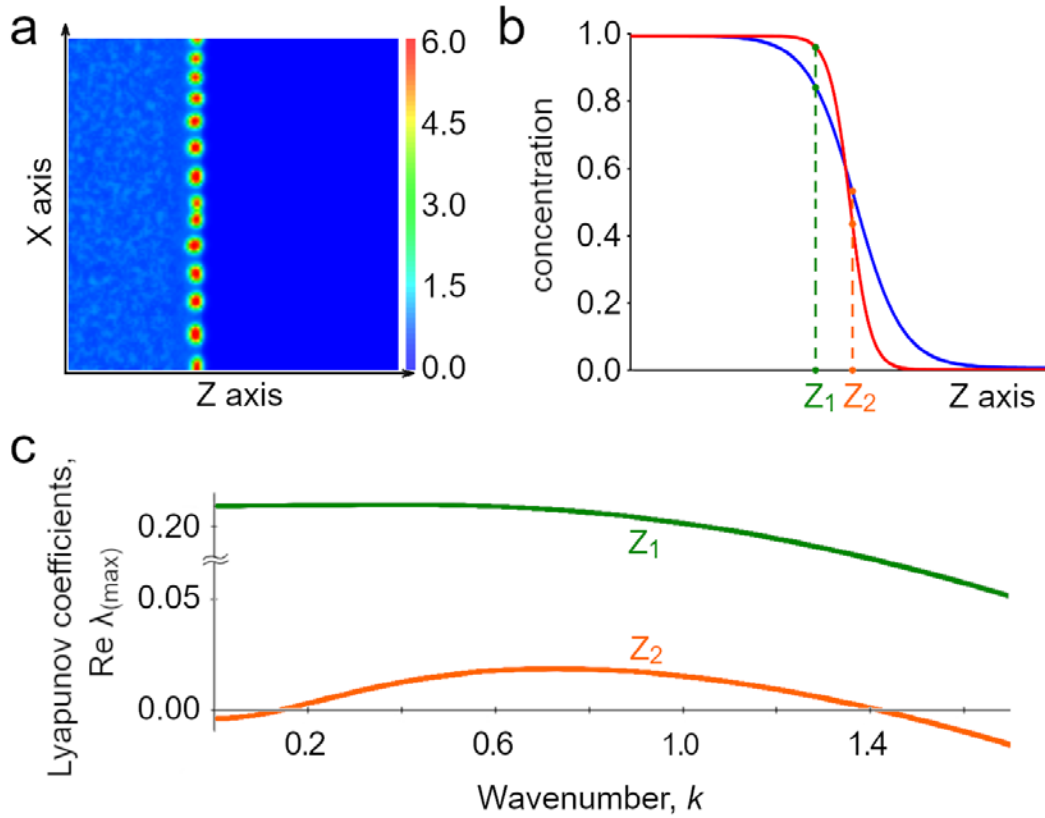


Supplementary Figure 3. Surface curvature does not change the outcome of simulation of the nanopatterns.

In order to figure out the possible mechanism of nanostructures' thin layer formation above the secretion boundary (Supplementary Fig.4a), we performed the following analytical investigation. In order to calculate the Activator and Inhibitor concentrations along the Z axis at the early stages of patterning, we used formulas from our model exclusive of the $F(a,b)$ and $G(a,b)$ functions as describing short-range interactions:

$$\begin{cases} \frac{da}{dt} = D_a \Delta a - d_a a + s_a d \\ \frac{db}{dt} = D_b \Delta b - d_b b + s_b d \end{cases} \quad \text{Eq. 1}$$

where $s_a = s_b = 1$. The results of this simulation are shown in Supplementary Fig.4b. This panel shows that due to the difference in diffusion coefficients, the concentration gradient of the Activator and the



Inhibitor is not the same.

Supplementary Figure 4. Mechanism of nanostructures' thin layer formation. (a) Y-axis cross-section of simulation, showing that the patterns form only within a thin layer near the white noise boundary. Simulated concentration of reagents is color-coded on the right. (b) Simulated concentrations of the Activator (red) and the Inhibitor (blue) at the moment T_0 , before the start of pattern formation. Graphs are built based on Eq. 1. Z_1 and Z_2 mark different distances along the Z axis. (c) Dispersion relations for various distances shown at panel (b). Dispersion curves are built based on Eq. 2. Stable pattern formation cannot occur at Z_1 , but occurs at Z_2 .

In order to determine the possibility of the Turing pattern formation at different distances from the secretion area, we should measure the growth rates of generic perturbations by using the Lyapunov exponent¹². In the model we use, the simulation takes place in 3D, with constant anisotropic secretion. In this situation, the analysis should be done for a two-dimensional layer. In order to take into account the diffusion of reagents from other layers into the layer under analysis, we applied cubic coupling:

$$\begin{cases} \frac{da}{dt} = m_a a + i_a b + D_a \Delta a + c_a a(a - c_a) \\ \frac{db}{dt} = m_b b + a_b a + D_b \Delta b + c_b b(b - c_b) \end{cases} \quad \text{Eq. 2}$$

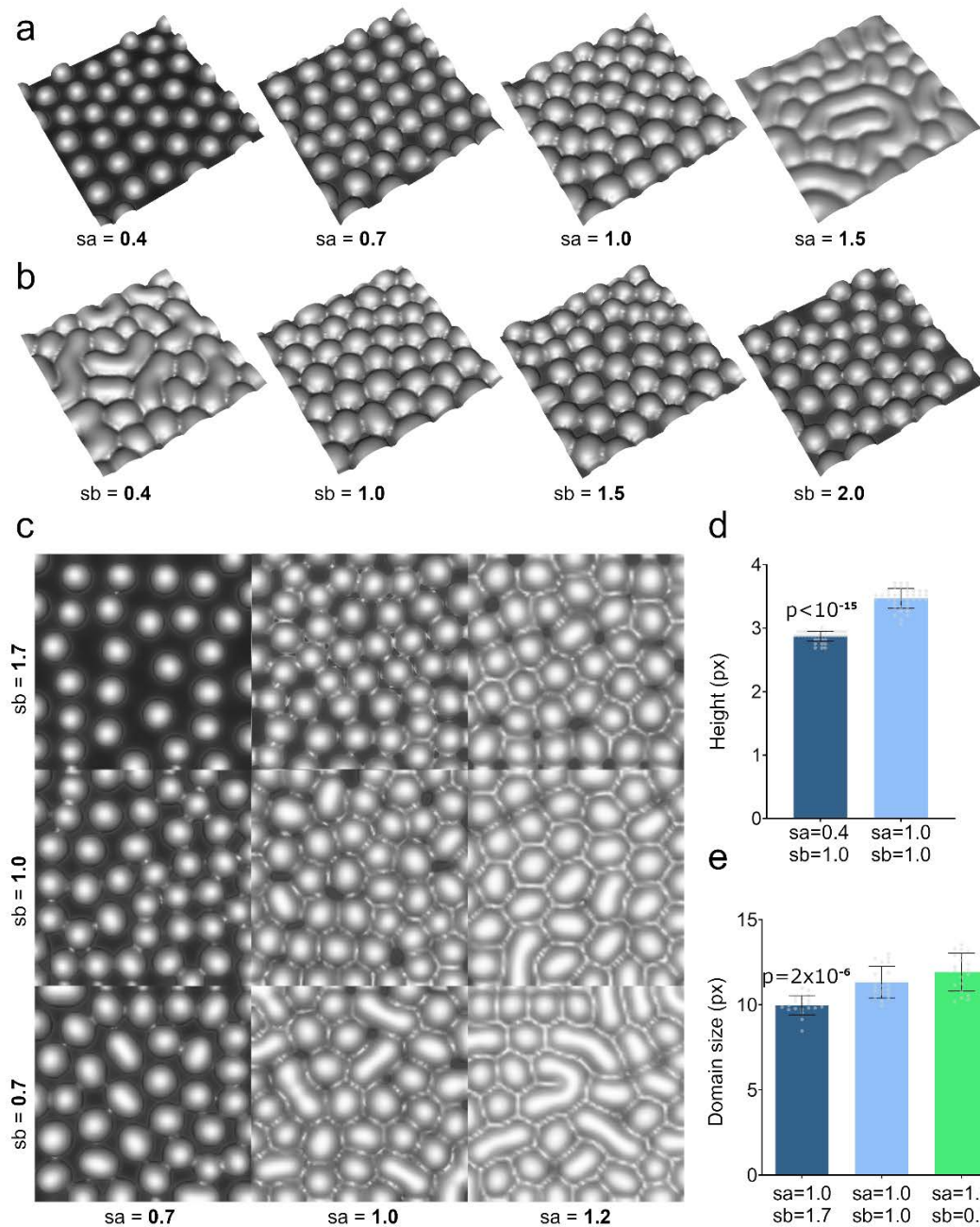
where: $m_a = a_a - d_a$, $m_b = i_b - d_b$, c_a and c_b – coefficients showing concentrations of the reagents, obtained from simulation of diffusion (Supplementary Fig.4b).

There is only one possible solution for the steady state, so that $Tr(\mathbf{A} - \mathbf{D})$ is negative: $a_0 = 0$, $b_0 = 0$, where

$$\mathbf{A} = \mathbf{J} \begin{pmatrix} m_a - 2c_a a + c_a^2 & i_a \\ a_b & m_b - 2c_b b + c_b^2 \end{pmatrix}_{a_0; b_0}; \quad \mathbf{D} = \begin{pmatrix} D_a & 0 \\ 0 & D_b \end{pmatrix} \quad \text{Eq. 3}$$

Taken into account that $Det(\mathbf{A} - \lambda \mathbf{I} - k^2 \mathbf{D}) = 0$, we can plot the dispersion relation of our simulation at different distances from the source. The plotted graphs (Supplementary Fig.4c) illustrate that up to the point Z_2 , patterning is impossible due to local instability⁴³. From this data, it can be concluded that the nanostructures indeed form within a thin layer at a certain distance from the site of secretion of the reacting reagents.

Simulations also show that a gradual increase in secretion levels of the Activator induces a gradual growth of the nanoprotusions, followed by their fusion (Supplementary Fig.5a).



Supplementary Figure 5. (a,b) Increased Turing Activator and decreased Turing Inhibitor lead to type-1 to type-2 nanocoating transition. 3D Turing modeling predicts that increase in the production/secretion of the Activator morphogen (sa) increases the size of nanostructures, and above a threshold induces transition into the nipple-to-ridge morphology (a). Reciprocally, separation of fused nanostructures into individual protrusions and reduction of their size is induced by increase in the production/secretion of the Inhibitor (sb) morphogen (b). (c) Patterns derived from Turing modeling combining different secretion levels of the Activator and Inhibitor morphogens. (d) Height of simulated 3D structures, obtained with different level of the sa variable show the trend similar to that seen in insect corneal nanostructures (WT and *k/d Retinin* (Fig. 2a, b) or type-1 vs. type-2 nanocoatings seen in different *Drosophilas* and correlating with the levels of Retinin (Fig. 1d, h)). (e) Size domain of simulated structures, obtained with different levels of the sb variable. Data in (d) and (e) are presented as mean $\pm$ SD. Statistical significance of the differences from the simulation with sa=1.0 and sb=1.0 was assessed by the Student's t-test (two-sided); n=3 independent experiments.

From this pattern, increasing secretion levels of the Inhibitor produces the opposite transition: disassembly of the nano-ridges into individual nanostructures, which further decrease in size and gradually disappear (Supplementary Fig. 5b). These simulations predict that decreased levels of the Inhibitor, or increased levels of the Activator, may stay behind the transition from the type-1 (individual nipple-like) nanocoatings to the type-2 (merged nipples) nanocoatings seen in different *Drosophila* species, leading us to identify Retinin as the Activator and waxes as the Inhibitor behind formation of the corneal nanocoatings.

In order to find the limits of the Turing model we used the equation from our previous work⁴:

$$D \propto f \omega^2 / 4\pi^2 \quad \text{Eq. 4,}$$

where D is the diffusion coefficient in cm^2/sec , and f is the constant of reaction rate in sec^{-1} . Permitting the bionanocoating structures to vary in their diameter from 50nm to 500nm, this creates the parameter space of $D \in [f * 6*10^{-11}; f * 6*10^{-13}]$, in which the nanocoatings are permitted to form (white diagonal stripe in Supplementary Fig.6a). Outside this area, the probability of bionanocoatings to form through the reaction-diffusion mechanism trends to zero (Supplementary Fig.6a, red hatching). This analysis provides the theoretical assessment of the parameter space in which bionanostructures can be formed by the reaction-diffusion mechanism.

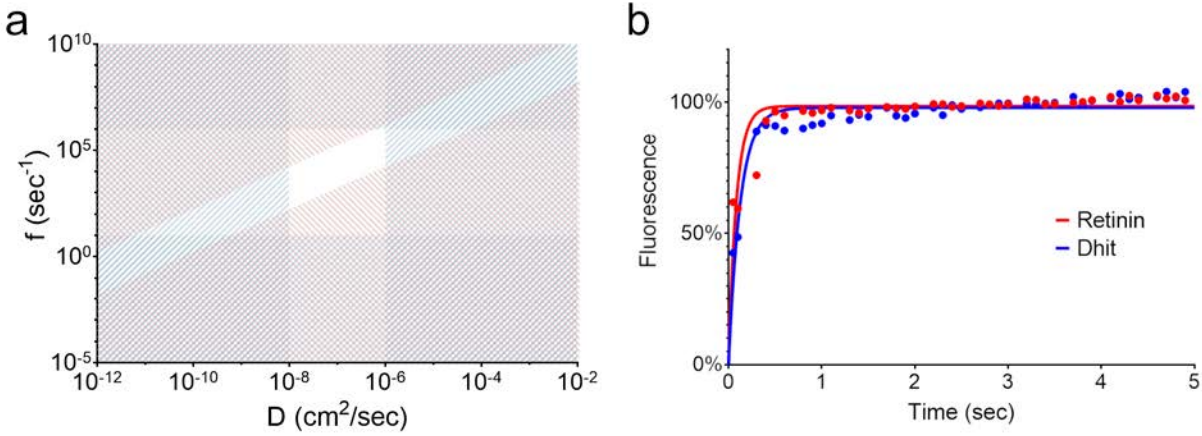
Identification of activator's and inhibitor's nature helps us to determine the limits of the D and f constants characteristic for the insect corneal nanocoatings. For the lower limit of D , we take $10^{-8} \text{ cm}^2/\text{sec}$, discovered for liquid crystals⁴⁴ and colloids⁴⁵. Such a limit was chosen, since both in the developing eye⁴⁶ and in the synthesis of artificial nanocoatings (see Methods), we deal with highly concentrated and complex solutions. We note, however, that even lower diffusion coefficients are conceivable (see our earlier work⁴ for these considerations). For the other boundary, we take protein diffusion rate in water, taking Lysozyme (14kDa) $D_{\text{water}} = 1.13 * 10^{-6} \text{ cm}^2/\text{sec}$ (ref⁴⁷) and GFP (27kDa) $D_{\text{water}} = 8.7*10^{-7} \text{ cm}^2/\text{sec}$ (ref⁴⁸) as the two approximations to Retinin (18kDa), producing ca. $10^{-6} \text{ cm}^2/\text{sec}$ as the upper limit of D .

In order to measure the reaction rate constant f , we applied the Thioflavin T (ThT) fluorescence method. ThT binds to proteins containing β -sheets composed of minimally four β -strands, which is followed by fluorescence enhancement of the fluorophore³⁴. Initially disordered, Retinin folds and interacts with ThT upon interaction with waxes (see Fig.3d and Methods). As controls we took the β -sheet-containing Dhit³¹ which does not interact with waxes (see Fig.3).

The resultant curves of the fluorescence increase kinetics are essentially identical (Supplementary Fig.6b). This allows us to conclude that the rate of the Retinin-wax interaction is faster or equal to the rate of the interaction of thioflavin with the β -sheet-containing proteins. As a vast molar excess of lanolin over Retinin was used in the experiment, we then can approximate the lower limit of the Retinin-wax reaction rate constant f with the unimolecular reaction,

$$f = \ln 2 / t_{1/2} \quad \text{Eq. 5}$$

Using the half-time of reaction from the experimental data (Supplementary Fig.6b), $t_{1/2} = 0.058$ sec, we obtain $f = 12$. Therefore, we can conclude, that reaction rate constant f is not less than 10 sec^{-1} . For the upper limit of f , we take 10^6 sec^{-1} characteristic for fast enzymatic reactions⁴⁹.

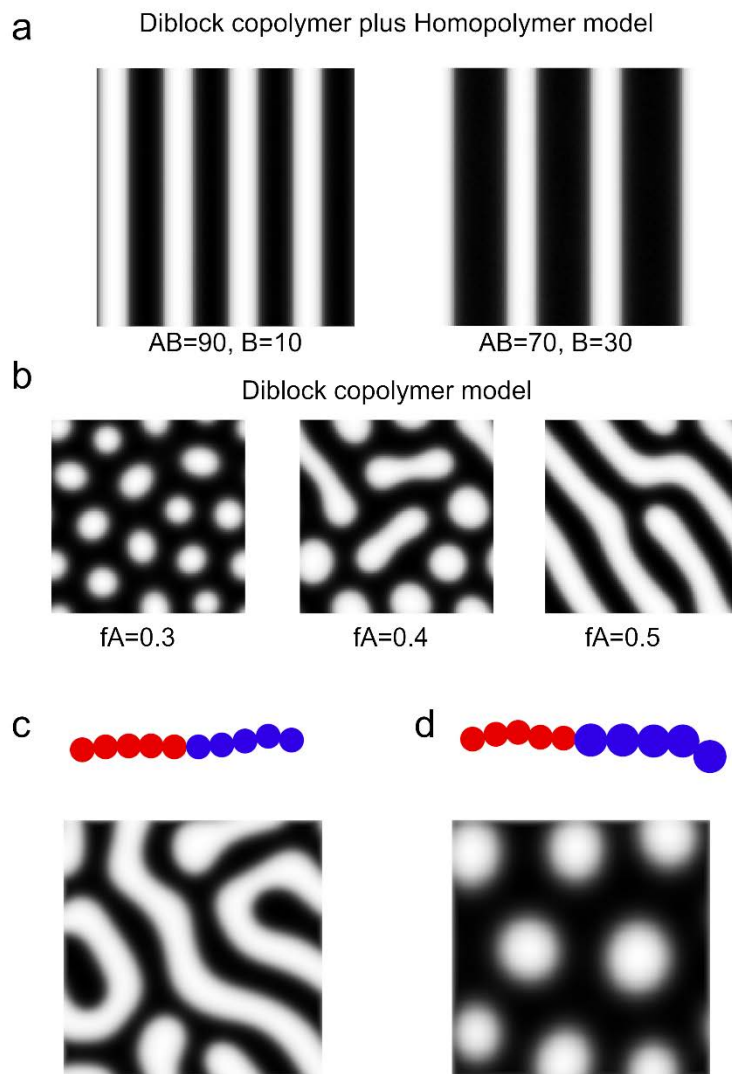


Supplementary Figure 6. (a) The parameter space defined by the diffusion coefficient D and the reaction rate constant f , in which the nanocoatings are allowed to form through the reaction-diffusion mechanism (the white diagonal stripe). Outside this area, the probability of bionanocoatings to form through the Turing mechanism trends to zero (red hatching). This theoretically predicted area overlaps with the physically realistic parameter zone of D and f (light-colored box). (b) ThT fluorescence kinetics upon the interaction with Dhit (blue) or with the Retinin-lanolin mixture (red). Fluorescence values are normalized to the maximum (mean for the measurements in the period of 2 to 5 seconds); $n=2$ independent experiments. See text for experimental details.

These evaluations of the possible limits of the parameters D and f identify the physical window within the parameter space, within which the Retinin-wax interactions in the developing corneae take place (light-colored box in Supplementary Fig.6a). Importantly, this physical window overlaps with the parameter space in which bionanocoatings can form as theoretically predicted by the reaction-diffusion mechanism (white diagonal stripe in Supplementary Fig.6a). Thus, in the manner more expanded than in our prior analysis⁴, we conclude that corneal nanocoatings built through the interactions of Retinin and waxes may well form through the reaction-diffusion mechanism.

As an alternative to the Turing-based reaction-diffusion model, block copolymer (BCP) model can be considered to describe the formation of bionanocoatings. The BCP self-assembly mechanism is based on polymers containing segments with different physical properties driving phase separation, and has been widely employed in formation of artificial nanostructures⁵⁰. However, unless using polymers with very long chains (see e.g. ref⁵¹), the dimensions of the resultant structures is typically within the 1-10nm range⁵⁰. As the corneal nanocoatings we study are based on the interactions of Retinin with waxes, it is relevant to consider the examples when protein-polymer bioconjugate-based BCPs have been developed. Importantly, such BCPs produce nanostructures with the diameter of 20nm or lower⁵²⁻⁵⁴, >10-fold smaller than the bionanocoatings we study and artificially reproduce in our work. Further, in such a diblock copolymer model (where we assume existence of Retinin-wax conjugates as the diblock polymer and free wax as the homopolymer, following the strong molar excess of wax over Retinin in our admixtures)

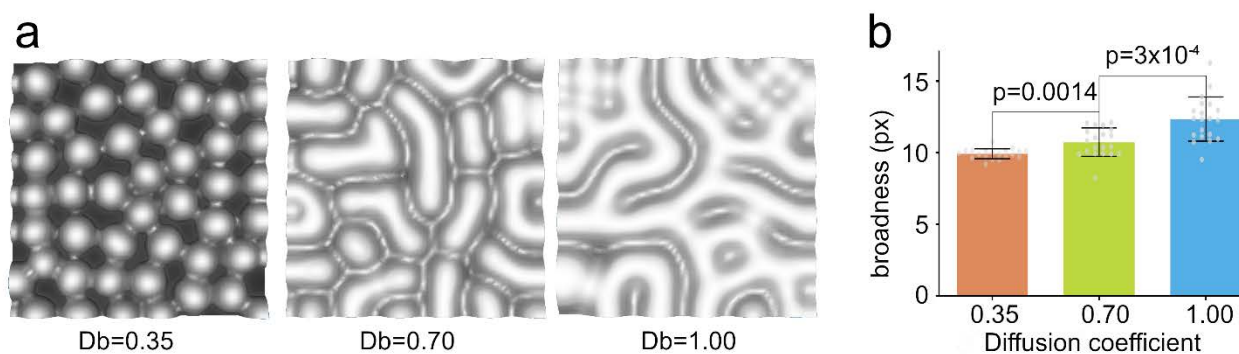
increasing amounts of the homopolymer in the mixture leads to increase in the domain size without change in the type of pattern⁵⁵ (Supplementary Fig.7a). This is in opposition to our experimental findings, where increased wax production – viewed as increased production of the Inhibitor morphogen in the Turing model – leads to fundamental changes in the nanopattern, both in insect corneae (Supplementary Fig.16a, central column, from bottom to top, quantified in Supplementary Fig.16b) and *in vitro* (Fig.4a, from right to left), which is accompanied by a decrease in the domain size. Thus, we find that this BCP model provides predictions not corresponding (in the sense of pattern type) and even contradicting (in the sense of the pattern size) the experimental observations. Turing modelling, in contrast, corresponds well to the experimentally observed findings (Supplementary Fig.5c, e and Supplementary Fig.16).



Supplementary Figure 7. Patterns obtained by different Diblock copolymer model simulations. (a) Lamellar structures, formed in the presence of 10% or 30% of homopolymer. (b) A diblock copolymer model predicts formation different patterns obtained by changes of proportions of polymer A (Retinin) and polymer B (wax). (c, d) Changes in the length of polymer B (in blue) not only changes the pattern from maze-type (c) to nipples-like (d) but also increases the domain size.

An alternative BCP model can be built, assuming certain pre-polymerization / pre-aggregation of the Retinin and wax monomers into building blocks, which would then be capable of phase separation to form nanostructures – a diblock copolymer model without homopolymer. If we assume existence of such pre-polymerized protein-lipid building blocks, we must note that such blocks are unlikely to be covalently linked or otherwise stabilized, as corneal Retinin in SDS-polyacrylamide gels migrates at a single band of ca. 25kDa, unlike the two other major corneal proteins Crystallin and Cpr72Ec, which display signs of significant cross-linking in the corneae (Supplementary Fig.12, also see²⁷). If we nevertheless assume formation of pre-polymerized yet relatively unstable Retinin-wax complexes as the starting material for the BCP-based assembly, resultant modelling can produce different patterns (Supplementary Fig.7b). In order to probe the efficiency of the diblock polymer model in recapitulating our experimental

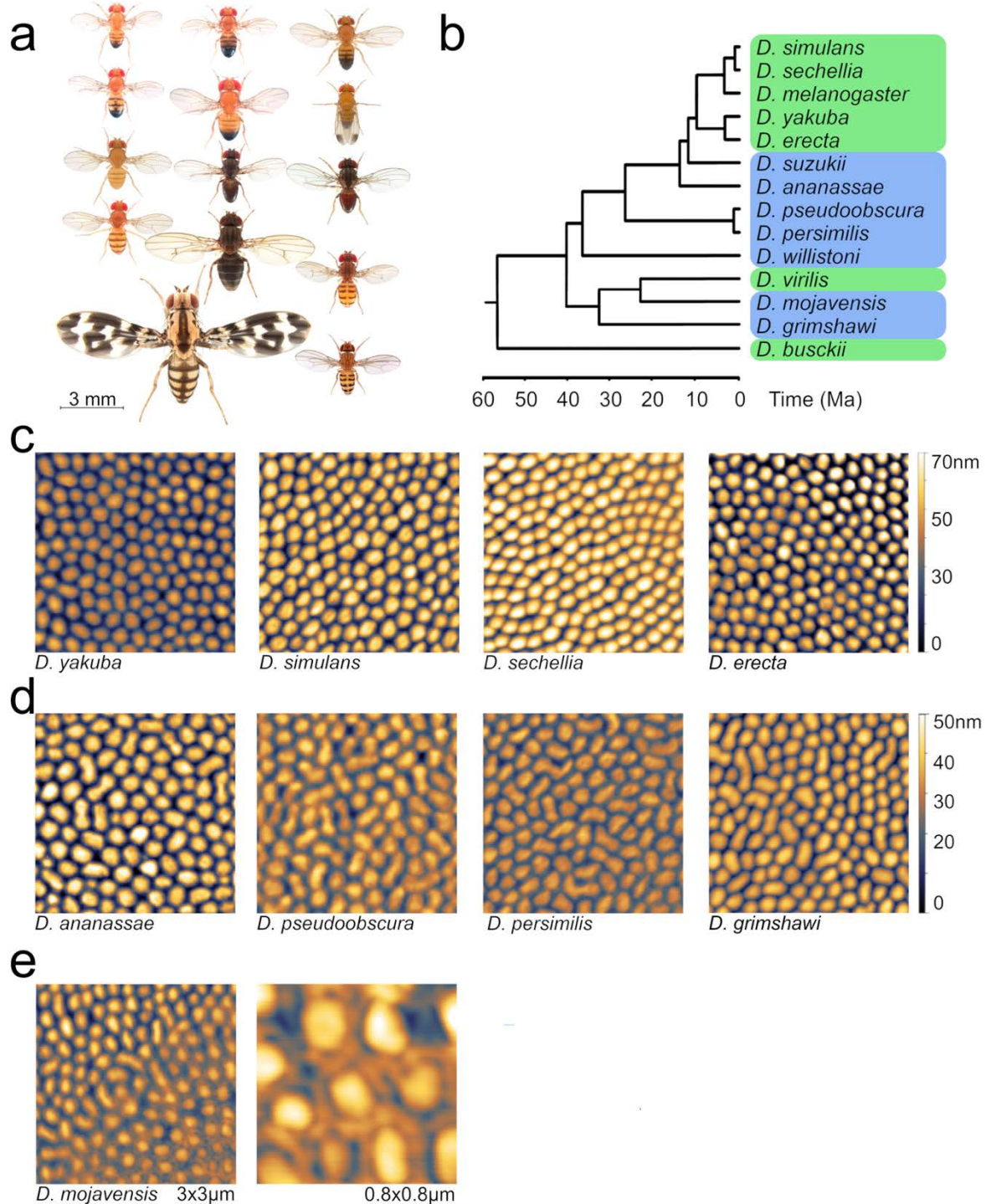
observations, we turn to the data presented on Supplementary Fig.17g, h, where waxes with different chain lengths were used, ranging from lanolin (C_{24-50}) to carnauba (C_{50-60}). This difference in the chain length will change the length of the hypothetical Retinin-wax copolymer (Supplementary Fig.7c,d). BCP modelling then predicts that this increase in the copolymer length will produce i) transition from the maze-type to nipple-type pattern, accompanied by ii) increase of the domain size of structures^{55,56} (Supplementary Fig.7c,d). The direction of the predicted pattern change agrees well with the experiment (Supplementary Fig.17g), however, experiment reveals the accompanied decrease in the domain size, opposite to the increase predicted by the BCP modelling (*cf.* Supplementary Fig.7c,d and Supplementary Fig.17g,h). Turing modelling, in contrast, corresponds very well to the experimentally observed findings (Supplementary Fig.8).



Supplementary Figure 8. (a) Increasing diffusion coefficient of the Turing Inhibitor leads to broader and gradually fusing nanostructures. 3D Turing modeling applied here is in a good agreement with the experimentally observed nanopatterns formed by admixing recombinant Retinin with waxes with different melting temperatures (see Supplementary Fig. 17f, g). Data in (b) are presented as mean $\pm$ SD. Statistical significance was assessed by the Student's t-test (two-sided), $n=3$ independent experiments.

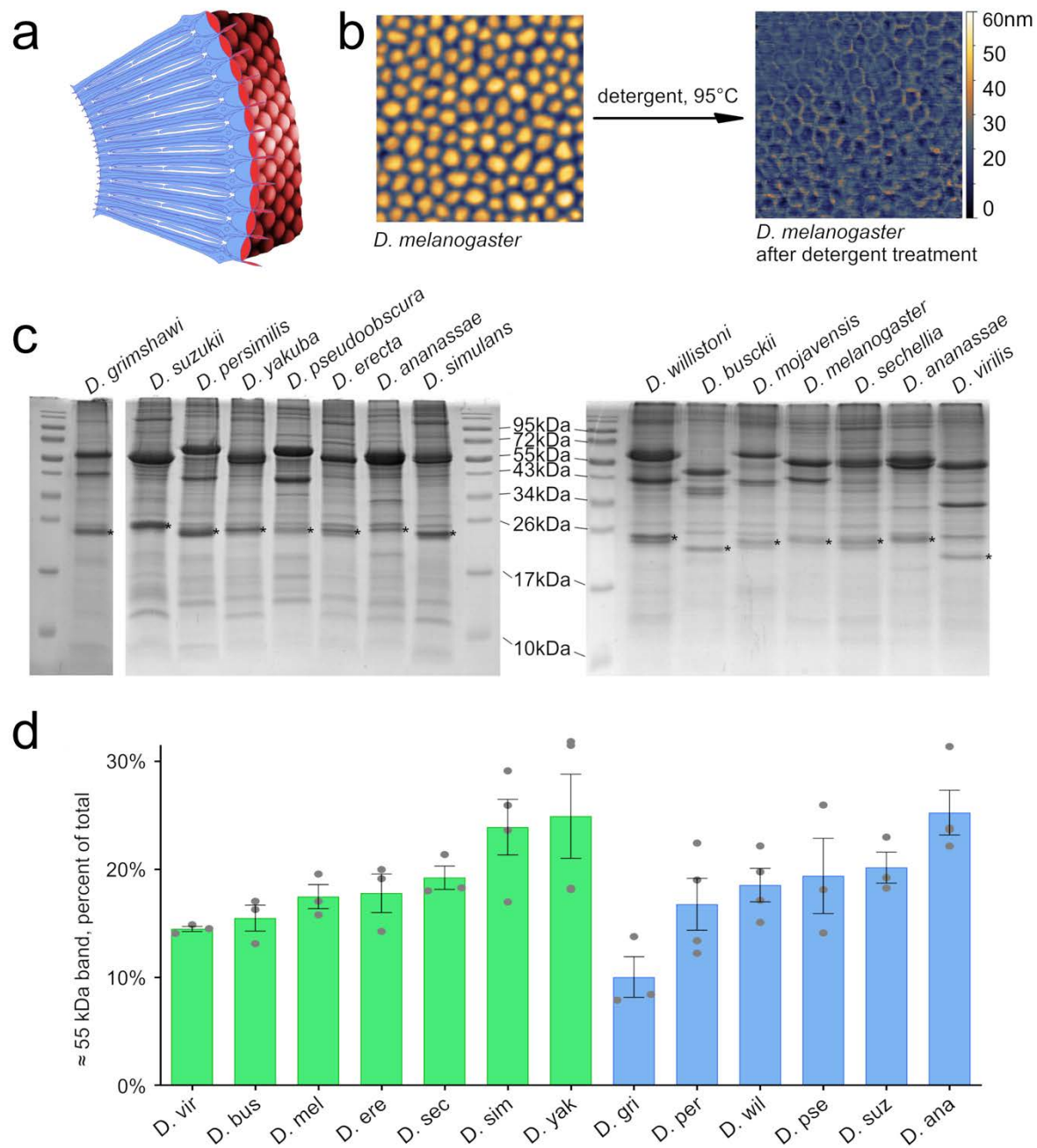
A modified Turing-based simulation model was created for the artificial nanocoatings. The model preserves the interaction principles and diffusion coefficients of the reagents, but modifies the starting parameters to accommodate the fact of immediate arrival of the reagents' concentrations to the fixed points upon *in vitro* admixtures, as opposed to the gradual secretion observed in insect corneae. Further, this model considers the gradual growth of the concentrations on the glass surface due to evaporation. To accommodate these changes, we added one more layer below the white noise (0.05 sizes of cube) for the d , equal to zero. This layer represents the glass, while the thin layer of the white noise on top of it represents the layer of colloidal particles interacting with the glass surface. In order to show the possibility of interaction between glass and the Retinin/lanolin-containing colloids, we measured the zeta potential of these colloids, which provided the value of -28mV. Since this value is lower than the zeta potential of glass (-23mV)⁵⁷, we can assume that these colloid particles will indeed interact with the glass surface.

Supplementary Note 2. Structure and composition of corneal nanocoatings across the genus *Drosophila*.

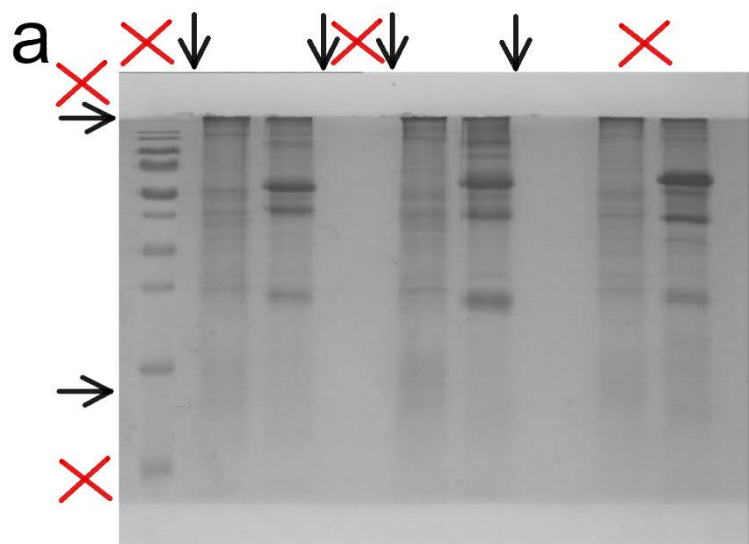


Supplementary Figure 9. (a, b) Fourteen *Drosophila* species (image courtesy of Nicolas Gompel, a) and their phylogenetic tree⁵⁸ (b) are characterized by two distinct types of corneal nanostructures (color-coded in green and blue). (c, d) Representative AFM images (3x3μm) of additional *Drosophila* species harboring type-1 nanocoatings (*D.yakuba*, *D.simulans*, *D.sechellia*) (c) and the type-2 nanocoatings (*D.ananassae*, *D.pseudoobscura*, *D.persimilis*) (d). (e) In *D.mojavensis*, harboring the type-2 nanocoatings, additional sub-nanostructures around the main structures can be noticed (magnification on the 0.8x0.8μm panel).

3 regions from 2 biologically independent animals were analyzed by AFM independently for each *Drosophila* species and gave similar results.



Supplementary Figure 10. (a) Scheme of separation of the eye material into retina (blue) and cornea (red) for subsequent proteomic analysis. (b) AFM analysis of *D. melanogaster* corneal surfaces before and after detergent treatment; the experiment was independently repeated 2 times with similar results. (c) SDS-PAGE of corneal preparations from different *Drosophila* species reveals a similar pattern of major proteins, including the ca. 25kDa protein present in all species and identified by mass-spectrometry as Retinin, and the ca. 55kDa protein; the experiment was independently repeated 3 times with similar results. (d) The abundance of the 55kDa corneal protein varies among *Drosophila* species without any correlation with the type of the nanocoatings. Data are presented as mean $\pm$ s.d.; n=3 independent experiments.



Supplementary Figure 11. Raw images representing the gel (a, corresponding to Fig.1e) and the membrane (b, upper panel) and the resulting Western blot image (b, lower panel) corresponding to Fig. 3b. Thermo Scientific PageRuler™ Prestained Protein Ladder (10 to 180 kDa) was used in both cases. Arrows and red crosses (a) and dashed lines (b) indicate how the gel and membrane were cropped for the final figures.

Supplementary Table 1. Mass-spectrometry analysis of the ca. 25kDa SDS-PAGE band from corneae of 12 *Drosophila* species.

species / genes (or loci)	MW	Percentage of total spectra	Percent coverage	Exclusive unique peptide count	Exclusive unique spectrum count	Description
<i>Drosophila virilis</i>						
GJ12785	19kDa	1.90%	76%	11	21	Drosophila Retinin-like protein
GJ22511	53kDa	0.48%	43%	16	26	Insect cuticle protein (Crystallin-like)
GJ12626	28kDa	0.36%	68%	13	20	Short chain dehydrogenase
GJ11018	21kDa	0.36%	58%	7	16	Retinophilin-like
GJ15754	22kDa	0.36%	53%	10	18	Tryparedoxin peroxidase
<i>Drosophila busckii</i>						
ALC43964	18kDa	0.71%	48%	8	13	Drosophila Retinin-like protein
XP_017848804	21kDa	0.46%	67%	18	31	ATP synthase delta
ALC43970	28kDa	0.45%	76%	15	28	Photoreceptor dehydrogenase
ALC39220	27kDa	0.35%	57%	13	24	C-type lectin
ALC44225	27kDa	0.30%	49%	11	18	Mitochondrial ATP synthase, B chain
<i>Drosophila melanogaster</i>						
CG13057	20kDa	1.70%	73%	9	22	Retinin
CG4899	28kDa	0.57%	66%	13	28	Photoreceptor dehydrogenase
CG3481	28kDa	0.50%	57%	15	27	Short chain dehydrogenase
CG8189	27kDa	0.42%	62%	17	24	ATP synthase delta
CG4784	26kDa	0.40%	46%	18	24	Cuticular protein 72Ec
<i>Drosophila erecta</i>						
GG15991	20kDa	1.50%	48%	10	103	Drosophila Retinin-

						like protein
GG13462	28kDa	1.10%	88%	28	79	Short chain dehydrogenase
GG13459	52kDa	0.41%	43%	19	29	Insect cuticle protein (Cuticular protein 72Ec-like)
GG15409	27kDa	0.38%	58%	15	27	Mitochondrial ATP synthase, B chain
GG11119	27kDa	0.38%	78%	15	27	NADP-dependent 3-hydroxy acid dehydrogenase
<i>Drosophila yakuba</i>						
GE19556	20kDa	2.20%	77%	12	24	Drosophila Retinin-like protein
GE22908	28kDa	1.00%	81%	22	48	Photoreceptor dehydrogenase
GE19037	28kDa	0.51%	86%	16	26	Alcohol dehydrogenase
GE20875	27kDa	0.38%	52%	14	23	Mitochondrial ATP synthase, B chain
GE16554	26kDa	0.34%	82%	15	21	3-ketoacyl-(acyl-carrier-protein) reductase
<i>Drosophila simulans</i>						
GD14627	18kDa	1.80%	55%	12	24	Drosophila Retinin-like protein
GD12481	30kDa	0.60%	71%	16	36	Short chain dehydrogenase
GD12478	52kDa	0.57%	51%	22	36	Insect cuticle protein (Cuticular protein 72Ec-like)
GD23968	28kDa	0.41%	86%	17	25	Alcohol dehydrogenase
GD23798	59kDa	0.32%	30%	14	19	Insect cuticle protein (Crystallin-like)
<i>Drosophila sechellia</i>						
GM25623	20kDa	1.80%	51%	12	24	Drosophila Retinin-like protein
GM24411	28kDa	0.93%	83%	21	47	Short chain

						dehydrogenase
GM24409	52kDa	0.61%	51%	23	38	Insect cuticle protein (Cuticular protein 72Ec-like)
GM26665	55kDa	0.57%	54%	20	35	Insect cuticle protein (Crystallin-like)
GM18074	26kDa	0.49%	60%	17	32	C-type lectin
<i>Drosophila pseudoobscura</i>						
GA12009	20kDa	1.80%	78%	16	32	Drosophila Retinin- like protein
GA18512	28kDa	0.72%	85%	21	43	Photoreceptor dehydrogenase
GA17214	28kDa	0.41%	83%	17	27	Alcohol dehydrogenase
GA18097	23kDa	0.35%	70%	16	24	ATP synthase delta
GA20881	28kDa	0.35%	58%	15	21	Mitochondrial ATP synthase, B chain
<i>Drosophila ananassae</i>						
GF24187	21kDa	2.30%	81%	14	26	Drosophila Retinin- like protein
GF24389	28kDa	1.60%	85%	27	59	Short chain dehydrogenase
GF14888	28kDa	0.49%	81%	21	35	Alcohol dehydrogenase
GF24387	51kDa	0.45%	40%	19	28	Insect cuticle protein (Cuticular protein 72Ec-like)
GF14419	26kDa	0.41%	57%	16	25	C-type lectin
<i>Drosophila persimilis</i>						
LOC6597216	23kDa	0.41%	70%	17	26	ATP synthase delta
LOC6593284	28kDa	0.41%	83%	16	25	Alcohol dehydrogenase
LOC6602777	28kDa	0.38%	58%	15	24	Mitochondrial ATP synthase, B chain
LOC6588132	20kDa	0.36%	69%	13	19	Mitochondrial ATP synthase, D chain
LOC6603169	26kDa	0.36%	50%	13	23	C-type lectin
compared to <i>D.</i>						

<i>pseudoobscura</i>						
GA12009	20kDa	1.80%	78%	16	32	Drosophila Retinin-like protein
GA18512	28kDa	0.68%	85%	21	40	Photoreceptor dehydrogenase
GA17214	28kDa	0.38%	76%	15	23	Alcohol dehydrogenase
GA20881	28kDa	0.38%	58%	15	24	Mitochondrial ATP synthase, B chain
GA16905	28kDa	0.35%	47%	13	22	C-type lectin
<i>Drosophila willistoni</i>						
GK20840	20kDa	1.40%	77%	12	25	Drosophila Retinin-like protein
GK20840	28kDa	0.92%	88%	23	43	Short chain dehydrogenase
GK18290	28kDa	0.47%	81%	22	32	Alcohol dehydrogenase
GK15169	58kDa	0.46%	43%	20	35	Insect cuticle protein (Crystallin-like)
GK20818	39kDa	0.39%	59%	22	30	Insect cuticle protein (Cuticular protein 72Ec-like)
<i>Drosophila suzukii</i>						
LOC108011322	20kDa	2.30%	51%	11	22	Drosophila Retinin-like protein
LOC108009320	28kDa	0.07%	25%	5	5	14-3-3ζ
CRX36800	14kDa	0.04%	22%	2	3	Cytochrome oxidase subunit II

Protein identity of the major corneal protein migrating at ca. 25kDa (see Fig.1g, Supplementary Fig.10c) from 12 species of the genus *Drosophila* was revealed by mass-spectrometry; data analysis was performed by the Scaffold software. For all investigated species except *D.persimilis*, Retinin emerges as the dominant component of the band. The failure to identify Retinin in *D.persimilis* is an artefact resulting from the genome sequencing error of this fruit fly, as usage of the genomic sequences from *D.pseudoobscura* identifies Retinin as the dominant protein in the 25kDa band from *D.persimilis*.

For the identified proteins, their NCBI designations are provided in the first column and their predicted molecular weights – in the second column. Percentage of total spectra (third column) refers to the ratio of

the spectra associated with a given protein to the total spectrum count within the sample. Percent coverage (fourth column) refers to the peptide sequences of the identified protein in percentage to the overall amino acid sequence of the protein. Exclusive unique peptide count (fifth column) refers to the number of unique peptides identified. Exclusive unique spectrum count (sixth column) refers to the number of spectra attributed uniquely to the identified protein. The seventh column provides a short description of the identified proteins.

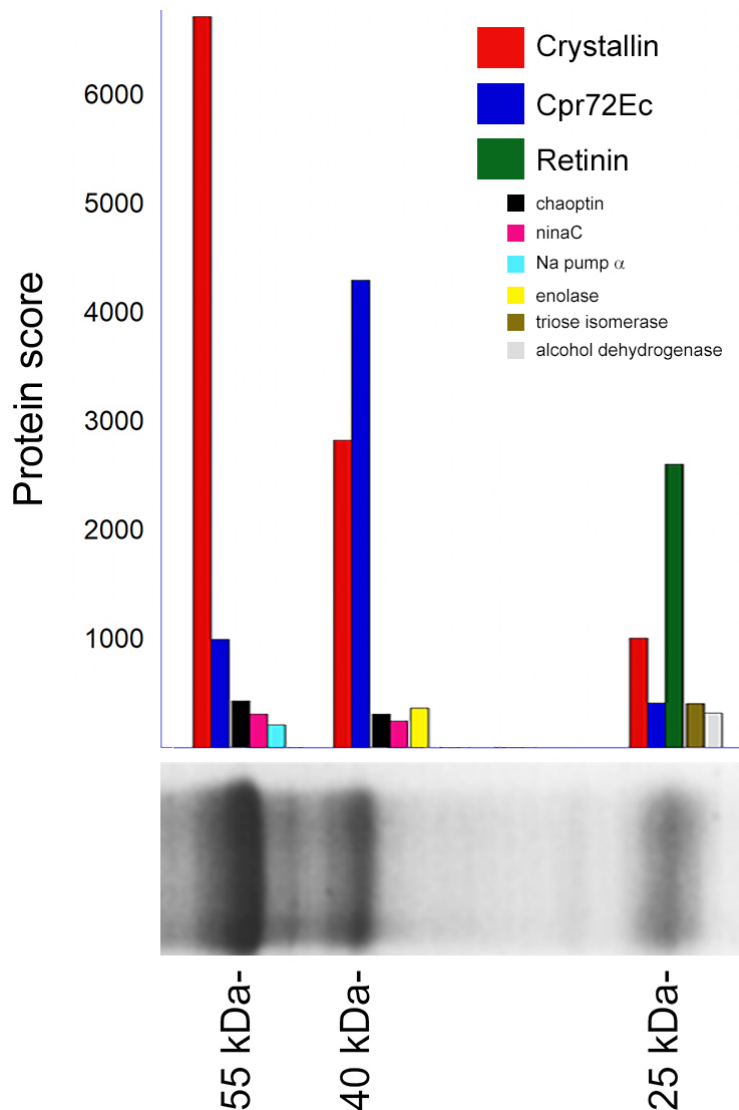
Supplementary Table 2. Mass-spectrometry analysis of corneal proteins from *D.melanogaster*.

Protein bands and respective genes	MW [kDa]	Protein score	Number of peptide spectrum matches	Percent coverage	Total number of peptides	Description
MAJOR BANDS						
≈55kDa						
<i>CG16963</i>	55.1	6725.77	218	43%	17	Crystallin
<i>CG4784</i>	50.5	996.12	30	21%	9	Cuticular protein 72Ec
<i>CG1744</i>	143.4	433.29	15	13%	11	chaoptin
<i>CG5125</i>	131.8	311.76	8	69%	7	ninaC, neither inactivation nor afterpotential C
<i>CG5670</i>	91.9	213.82	8	12%	8	Na pump α subunit
≈40kDa						
<i>CG4784</i>	50.5	4301.05	139	37%	16	Cuticular protein 72Ec
<i>CG16963</i>	55.1	2831.04	109	34%	11	Crystallin
<i>CG17654</i>	46.6	364.90	10	27%	8	Enolase
<i>CG1744</i>	130.6	310.79	13	13%	11	chaoptin
<i>CG5125</i>	131.8	247.97	6	63%	6	neither inactivation nor afterpotential C
≈25kDa						
<i>CG13057</i>	19.9	2610.73	104	43%	6	Retinin
<i>CG16963</i>	55.1	1007.99	30	31%	10	Crystallin

CG4784	50.5	411.60	13	22%	8	Cuticular protein 72Ec
CG2171	26.6	406.53	11	43%	8	Triose phosphate isomerase
CG3481	27.6	318.55	12	47%	8	Alcohol dehydrogenase
MINOR BANDS						
≈45kDa						
CG4784	50.5	2660.96	86	37%	16	Cuticular protein 72Ec
CG16963	55.1	2320.53	92	34%	11	Crystallin
CG5125	131.8	231.60	6	63%	6	neither inactivation nor afterpotential C
≈35kDa						
CG16963	55.1	1893.03	66	34%	10	Crystallin
CG4784	50.5	858.88	29	23%	10	Cuticular protein 72Ec
CG9914	34.8	539.58	15	40%	10	3-hydroxyacyl-CoA dehydrogenase
CG9277	50.1	465.18	13	24%	9	β-Tubulin at 56D
CG11154	38.0	281.23	9	27%	7	ATP synthase, β subunit
≈30kDa						
CG16963	55.1	1431.66	55	34%	11	Crystallin
CG4784	50.5	1002.16	30	22%	9	Cuticular protein 72Ec
CG4994	38.8	283.08	7	18%	5	mitochondrial phosphate carrier protein
CG9150	27.8	229.13	6	24%	4	steroid oxidoreductase activity
CG16944	32.9	227.06	6	23%	6	stress-sensitive B

Protein identity of the three major corneal proteins of *D.melanogaster* migrating at ca. 55kDa, 40kDa, and 25kDa (see Fig.1g) and some minor bands migrating at ca. 45kDa, 35kDa, and 30kDa (see the *D.melanogaster* lane in Supplementary Fig.10c for better visualization of the minor bands) revealed by mass-spectrometry; data analysis was performed by the Proteome Discoverer software. The ca. 55kDa band represents Crystallin; the ca. 40kDa band – Cuticular protein 72Ec (Cpr72Ec); the ca. 25kDa band –

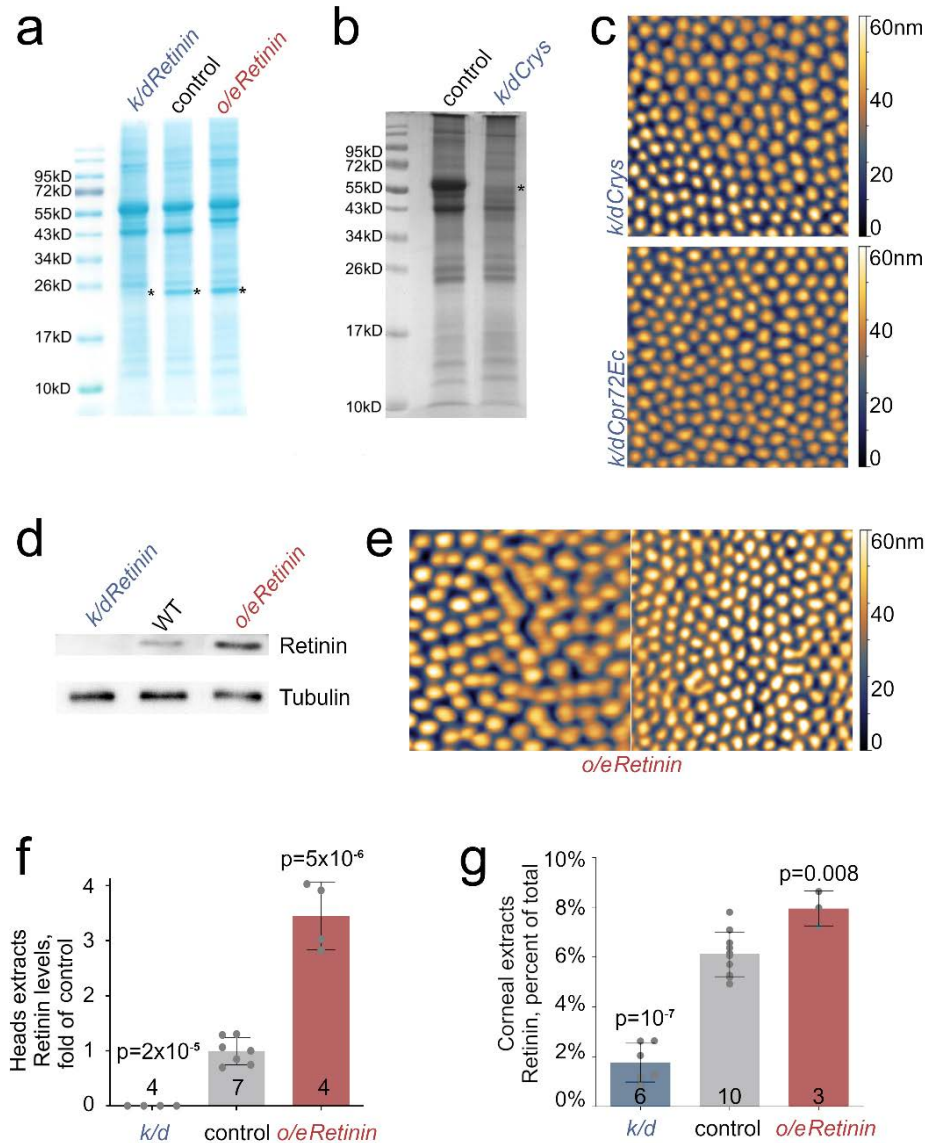
Retinin. The minor bands appear to contain contaminants and/or degradation products from Crystallin and Cpr72Ec. Minor proteins present in the bands are photoreceptor-resident proteins (e.g. ninaC, chaoptin, Na pump α subunit) or metabolic enzymes (e.g. Enolase, Alcohol dehydrogenase, Triose phosphate isomerase), likely being contaminants from the retina left after separation of corneae from the protein-rich underlying retinal material, despite the effort made to clean corneae from the retina (see Methods). Supplementary Fig.12 illustrates that the minor proteins identified are dramatically less abundant than the three major proteins, suggesting that they are likely contaminants from the underlying retina. For the identified proteins, their FlyBase designators are provided in the first column and their predicted molecular weights – in the second column. Protein score (column 3) refers to the sum of the ion scores of all peptides that were identified. Number of peptide spectrum matches (column 4) refers to the total number of identified peptide spectra matched for the given protein. The number of peptide spectrum matches value may be higher than the number of peptides identified for high-scoring proteins because peptides may be identified repeatedly. Percent coverage (fifth column) refers to the peptide sequences of the identified protein in percentage to the overall amino acid sequence of the protein. Total number of peptides (column 6) provides the total number of distinct peptide sequences identified in the protein. The seventh column provides a short description of the identified proteins.



Supplementary Figure 12. Data for the major corneal bands from the Supplementary Table 2 were plotted to illustrate protein abundance in each of the bands. The content of the minor proteins is miniscule as compared to the three major corneal proteins, indicating that they are likely contaminants from the underlying retina. Cross-linking of corneal Crystallin and Cpr72Ec is the likely reason for the presence of these proteins in more than one band on the gel (ref. ²⁷).

Supplementary Note 3. Genetic manipulations of *D.melanogaster* corneal proteins.

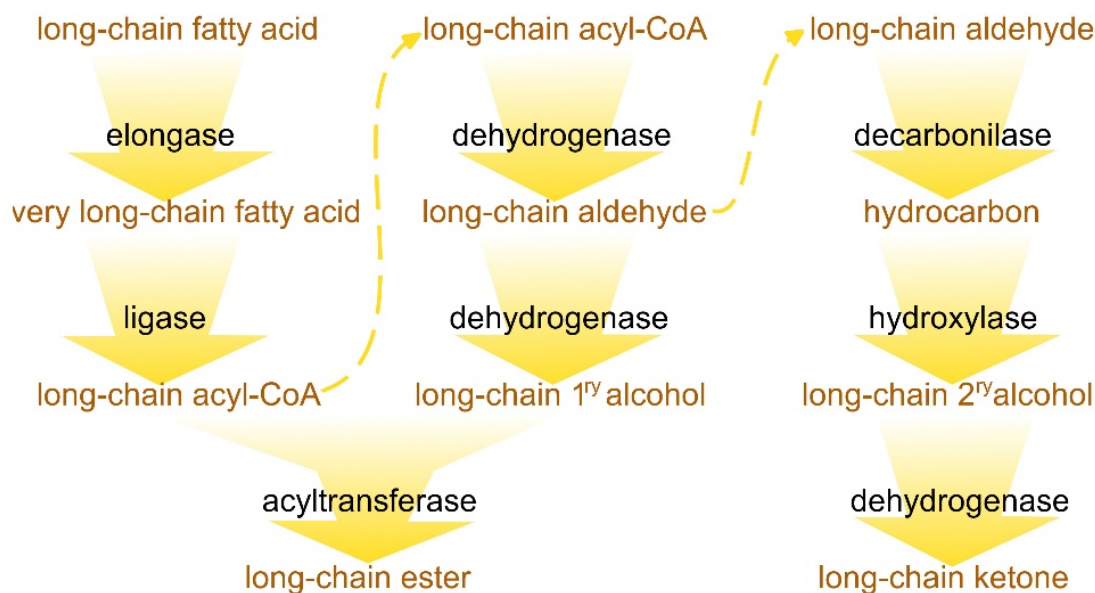
Further to the mass-spectrometry data, the assignment of the 55kDa band to Crys and of the 25kDa band to Retinin (Supplementary Fig. 13a, b) is confirmed by genetic knock-downs.



Supplementary Figure 13. (a, b) SDS-PAGE of corneal samples of *D.melanogaster* with downregulation (*k/d*) or overexpression (*o/e*) of Retinin (a) or *k/d* of Crys (b) confirms that the respective identities of the 25kDa and the 55kDa bands. Note that the abundance of Cpr72Ec (migrating at ca. 40kDa) in corneal samples may be influenced by Retinin and Crys. Similar results were obtained in 4 (a) and 2(b) independent experiments. (c) Representative AFM images (3x3μm) of Crys and Cpr72Ec *k/d* corneae. Similar results were obtained in 3 independent experiments. (d) Western blot of *Drosophila* heads extracts upon genetic manipulations of Retinin; the upper blot is with anti-Retinin, and the lower – with the anti-tubulin antibodies. Similar results were obtained in 4 independent experiments. (e) Representative AFM images (3x3μm) of corneae upon overexpression of Retinin. Similar results were obtained in 5 independent experiments. (f) Quantification of Retinin levels from Western blots of head extracts. (g) Quantification of Retinin levels in corneae detected by Coomassie staining (as in a). Data in (f) and (g) are presented as mean ± s.d. Statistical significance of the differences from the wild-type genotype (control) was assessed by the Student's t-test (two-sided); n (independent experiments) is indicated on each bar.

To assess the degree of *Retinin* down- and up-regulation, we performed Western blotting after SDS-PAGE of total head extracts from different genotypes, using antibodies we have generated against *D.melanogaster* Retinin (Supplementary Fig.13d,f). In addition, we performed Coomassie staining after SDS-PAGE of corneal material from different genotypes (Supplementary Fig.13a,g). Both means of analysis confirm a strong reduction in Retinin levels after RNAi-mediated downregulation of the gene. Overexpressions of *Retinin* are also detected. However, while overexpression increases Retinin levels in the whole-head samples, only a small portion of this overexpressed protein makes it to the corneae (Supplementary Fig.13a,d,f,g).

Supplementary Note 4. Genetic manipulations of the wax biosynthetic pathway in *D.melanogaster*.



Supplementary Figure 14. Scheme of the wax biosynthesis pathway, indicating intermediate products and the enzymes involved (ref. ²⁷⁻²⁹).

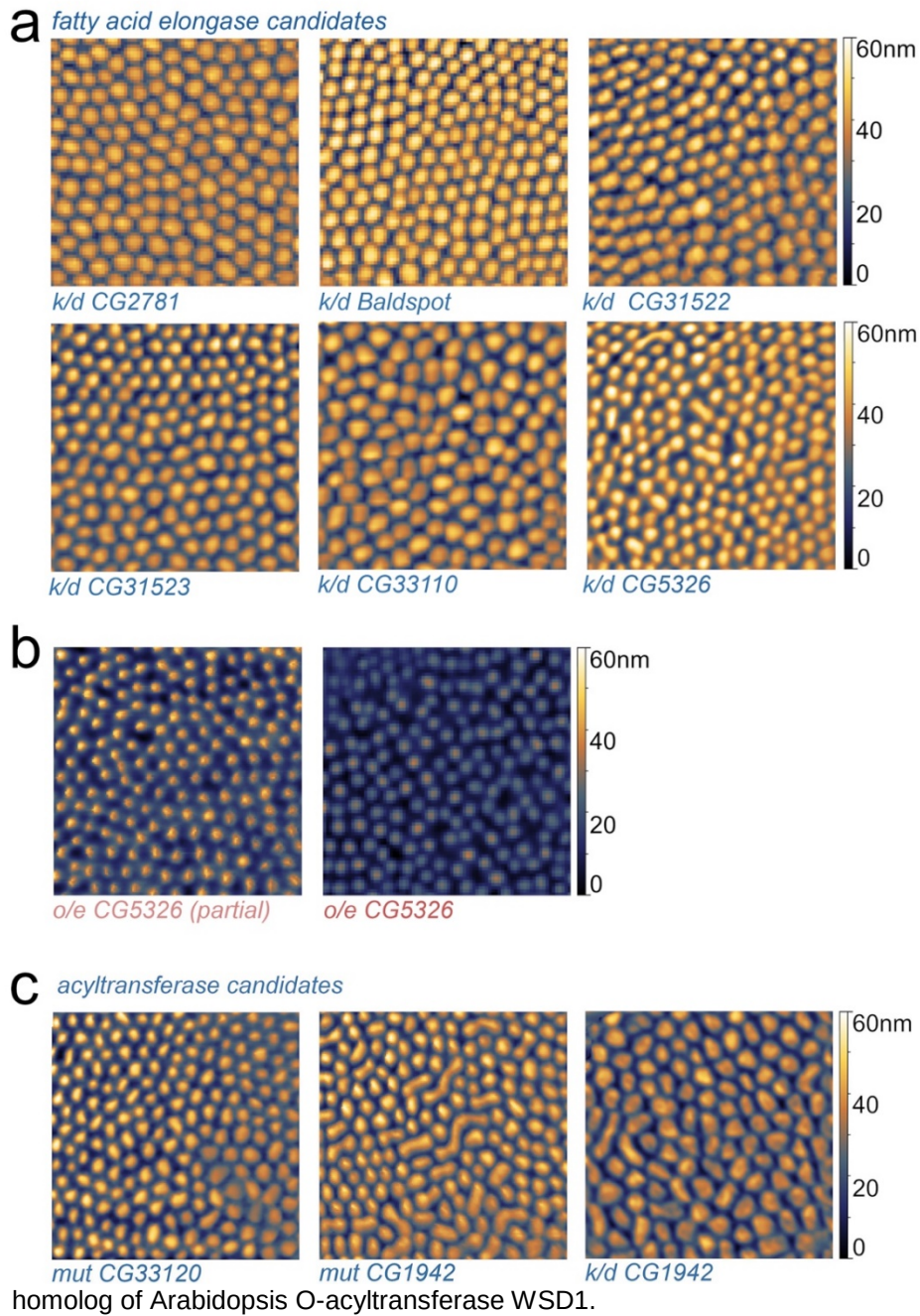
Supplementary Table 3. Candidate long-chain fatty acids elongases in *D.melanogaster*.

Gene	Predicted function	Tissue-specific expression level		
		High	intermediate	Low
CG27811	1,3-beta-D-glucan synthase activity	crop, hindgut, carcass, head	fat body	eye , midgut, malpighian tubules, heart, ovary
CG31523	very long-chain fatty acid metabolism (>C22)	crop, hindgut, midgut, fat body, heart, carcass,	head , inseminated female spermatheca	eye , brain, thoracic-abdominal ganglion, salivary gland, malpighian tubules, virgin female spermatheca, ovary, male accessory gland
CG3971/Baldspot	spermatogenesis	crop, hindgut, thoracic-abdominal ganglion	head , brain, midgut, malpighian tubules, salivary gland, virgin female spermatheca	eye , fat body, heart, ovary, testis, carcass, inseminated female spermatheca
CG33110	Unknown		head , carcass	eye , male accessory gland

CG5326	neurogenesis		crop, male accessory gland	head, eye , ovary, carcass
CG31522	very long-chain fatty acid metabolism (>C22)	crop, hindgut, thoracic-abdominal	ganglion, midgut	eye , brain, malpighian tubules, fat body, salivary gland, heart, virgin female spermatheca, inseminated female spermatheca, male accessory gland, carcass

BLAST-identified *Drosophila* homologs of the human fatty acid elongase converting long-chain fatty acids into very long-chain fatty acids at the beginning of the wax biosynthetic pathway (see Supplementary Fig. 14) with reported expression in the head and/or eye tissue. Gene name/symbol, predicted function, and tissue-specific expression are shown. Expression data were obtained from Flybase.org and FlyAtlas database.

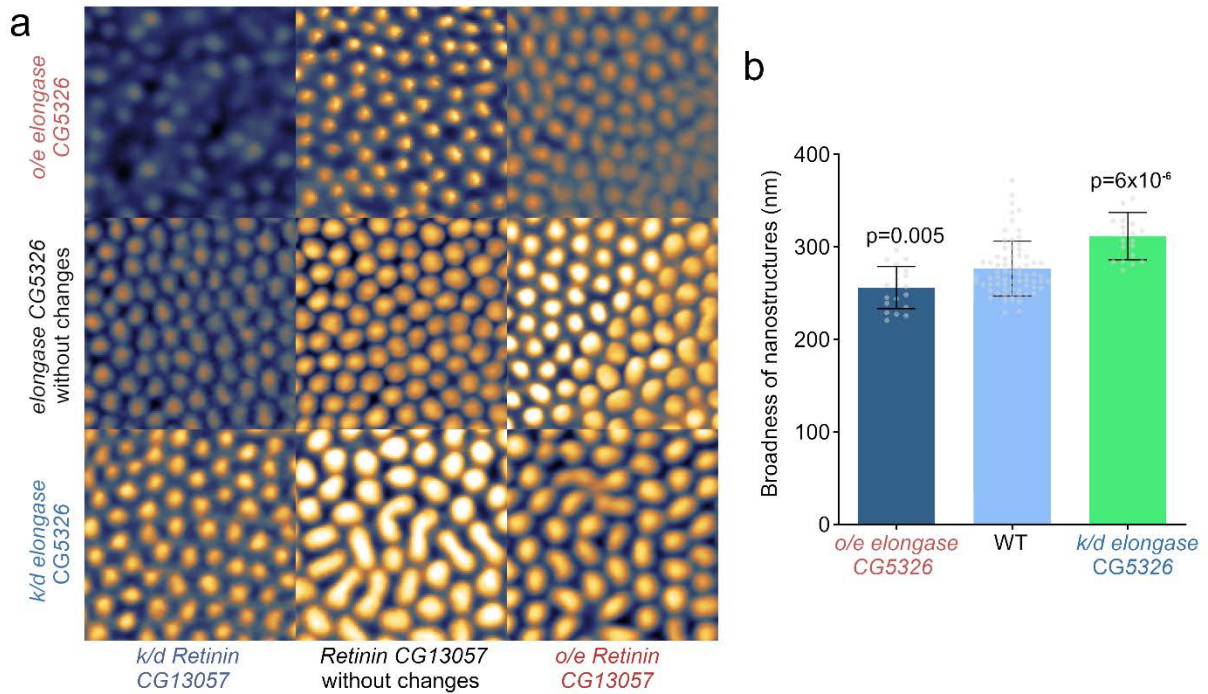
We identified only two candidates for acyltransferase activity in *Drosophila*: CG1942 as the homolog of the mammalian long-chain-alcohol O-fatty-acyltransferases AWAT1/AWAT2; and CG33120 as the



Supplementary Figure 15. (a, b) Representative AFM images (3x3μm) of corneal phenotypes resulting from genetic manipulations of the six *Drosophila* fatty acid elongase candidates: knock-down (*k/d*) of *CG2781*, *Baldspot*, *CG31522*, *CG31523*, *CG33110* and *CG5326*, as well as different levels of overexpression (*o/e*) of *CG5326* (using *spa-Gal4* in the left, and *GMR-Gal4* in the right panel). Similar results were obtained in 2 independent experiments. (c) Representative AFM images (3x3μm) of corneal samples

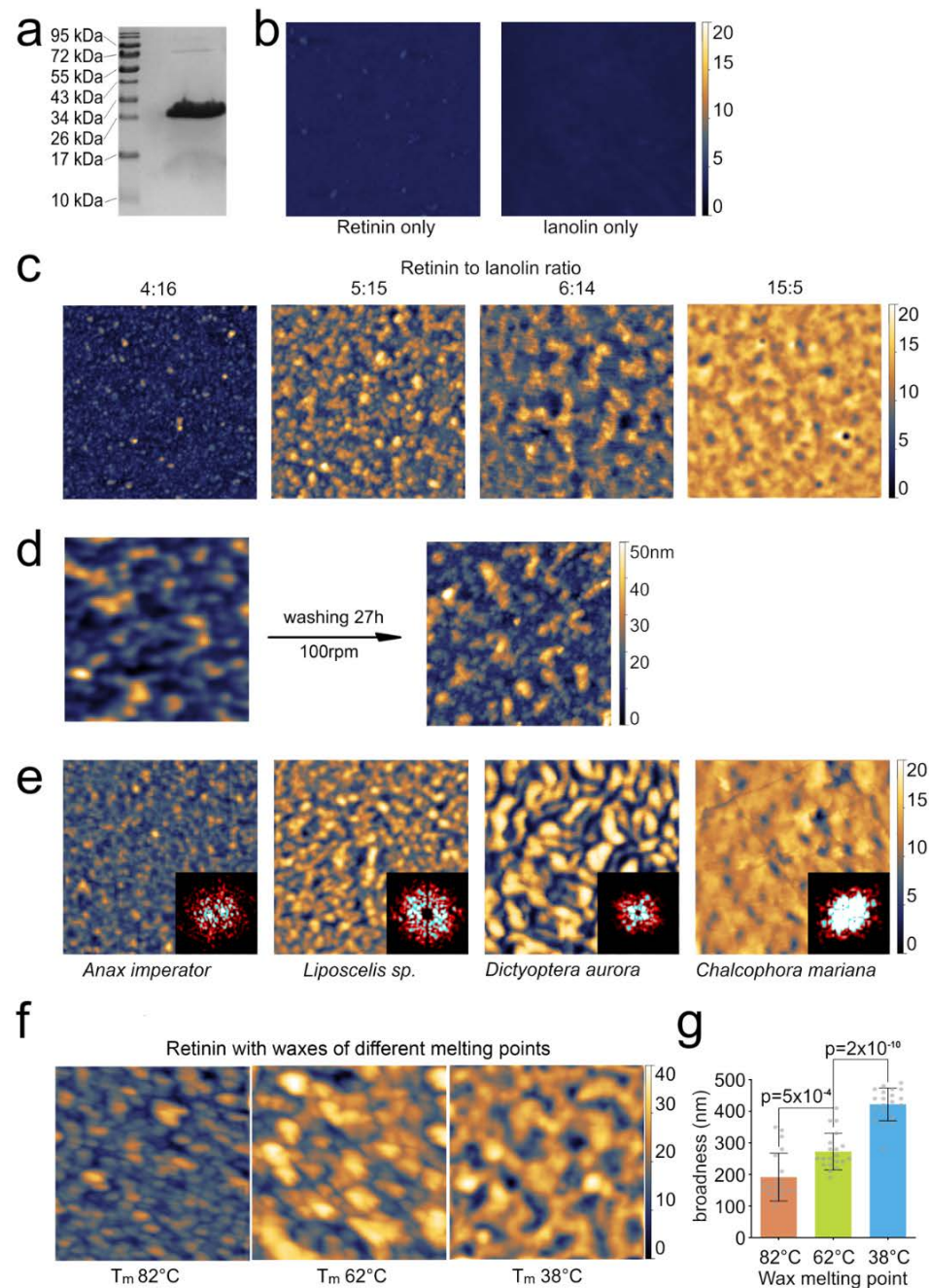
from *D.melanogaster* mutant for *CG33120* or *CG1942*, or *k/d* for *CG1942* – the acetyltransferase candidates. Similar results were obtained in 2 independent experiments.

Supplementary Note 5. Genetic interactions between *Retinin* and *CG5326* in formation of corneal nanocoatings.



Supplementary Figure 16. (a) Analysis of corneal nanocoatings from *D.melanogaster* overexpressing (o/e) or downregulating (k/d) *elongase* CG5326 and/or *Retinin*. Representative AFM images (2x2μm) are shown. Similar results were obtained in 3 independent experiments. Compare with Supplementary Fig. 4c: both (experimental and simulated data) reveal three principal patterns of nanocoatings: degraded small-nipples type, the nipple-only pattern (type-1), and the nipple-to-ridge pattern (type-2). (b) Changes in the broadness of corneal nanostructures, visible in transition from overexpression to down-regulation of *elongase* CG5326. Data presented as mean ± SD. Statistical significance of the differences from the wild-type genotype was assessed by the Student's t-test (two-sided); n=3 biologically independent animals.

Supplementary Note 6. Composition, stability and functionality of artificial nanocoatings.



Supplementary Figure 17. (a) SDS-PAGE of recombinant Retinin produced as a clean soluble protein. (b) Retinin alone or lanolin alone do not form nanocoatings. (c) Single-layer application of the Retinin:lanolin admixtures at the indicated proportions. (d) AFM images (4x4μm) of the artificial nanocoatings before and after 27h of washing in the water. (e) Different types of corneal nanocoatings seen in different insects. The color scale shown next to the images indicates the surface height (in nm). Inserts show Fourier transform spectra of the AFM images. (f) AFM images (2x2μm) of artificial nanocoatings obtained using different waxes and Retinin (see Methods), showing difference of patterns. For a-f, similar results were obtained in 2 independent experiments. (g) Broadness of nanostructures from panel d. Data are present as mean ± s.d., n=20 measurements from 2 independent experiments. Statistical significance of the differences was assessed by the Student's t-test (two-sided).

We provide the following considerations as an explanation of how the exact formulations of the synthetic nanocoatings lead to the specific functional properties.

As explained in the main text, increases in the Retinin to wax ratio lead to a morphological change of the nanocoatings, from nipple-like structures to ridges and next to a dimpled pattern (Fig. 4a, Supplementary Fig. 17c). Application of the identical second layer admixture increases the height of the structures without significantly affecting the type (Supplementary Fig.17c, Fig. 4a). Further applications of identical admixtures do not significantly change the height of the nanocoatings (data not shown). A significant increase in the height of nanostructures can be also achieved if Retinin alone is used in the second layering, but this is associated with a change in the nanocoatings type. Indeed, when Retinin alone is applied above the Retinin-lanolin layer, the initial pattern degenerates into shapeless piles (Fig.4d, protocol #4). Interestingly, Retinin can also be applied as a second layer on top of the waxed-only surface, resulting in nanostructures with closely packed nipple-like shapes of a relatively large height (Fig.4d, protocols #1 and #2).

The antireflection functionality of such resultant nanocoatings is in agreement with the generally accepted paradigms and solutions of Maxwell's equations², whereas the height of nanostructures is inversely proportional to the amount of reflected light, under the condition that they have a dense packaging and uniformity of sizes (Fig.4b,c).

The other functionality under study – the anti-adhesiveness in general and hydrophobicity in particular – depends on two major parameters. The first is the chemical composition of the structures: we have shown that the identical structures containing different waxes have different hydrophobicity, increasing in the series lanolin – beeswax – carnauba (data not shown). The second is the morphology of nanocoatings: similarly to what we describe for corneal nanocoatings in different species of *Drosophila* (Fig.1d and Fig.2d), the transition from the nipple-like structures to the maze-type pattern (and then further to the dimpled pattern) leads to a significant increase in the anti-adhesiveness (Fig.4c). We cannot exclude, however, that the increased anti-adhesiveness in this series of nanopatterns is not necessarily (or solely) the outcome of the pattern change, but also of different exposures of chemical groups of Retinin and wax resulting from the pattern rearrangement. This consideration agrees with the observation that addition of Retinin alone as the second layer significantly reduces the hydrophobicity of coatings. On the other hand, nipple-like structures demonstrate the greatest hydrophobicity of the structures we investigated, which correlates well with the morphology-based Cassie-Baxter model².

To sum up, we can conclude that the functionalities we ascribe to the artificial nanostructures are in agreement with the existing models of surfaces with antireflective, anti-adhesive, and hydrophobic properties.

Supplemental Methods contain the in-house generated MATLAB script used to calculate the parameters of corneal nanostructures:

```
%input file from Gwyddion should be in the same folder where you put the
%script, for many files switch on the iteration
clearvars()
data=tdfread('./1.txt');
heights=0;
for i = 1:3
    x=data.Profile_1_Profile_2_Profile_3(1:end,2*i-1);
    y=data.Profile_1_Profile_2_Profile_3(1:end,2*i);
    %% find absolute minimum of the curve - the first reference point
    [abs_min, abs_min_x]=min(y);

    %% find maximums to the left and to the right of the reference point
    [maxl, maxl_x]=max(y(1:abs_min_x));
    [maxr, maxr_x]=max(y(abs_min_x:end));

    %% find 2 potential secondary minimums
    %find two minimums following the maximums and choosing one of them
    %which is lower, no matter where it is
    %(only if none of the maximums is at the limit, then the choice is clear:
    if maxl_x ~= 1 & maxr_x ~= length(y(abs_min_x:end))
        [sec_minl, sec_minl_x]=min(y(1:maxl_x));
        [sec_minr, sec_minr_x]=min(y((abs_min_x+maxr_x):end));
        if sec_minl < sec_minr
            maxg=maxl;
            max_x=maxl_x;
            sec_min=sec_minl;
            sec_min_x=sec_minl;
        else
            maxg=maxr;
            max_x=abs_min_x+maxr_x;
            sec_min=sec_minr;
            sec_min_x=sec_minr_x+abs_min_x+maxr_x;
        end
    end
end
```

```

elseif maxl_x == 1
    [sec_minr, sec_minr_x]=min(y((abs_min_x+maxr_x):end));
    maxg=maxr;
    max_x=abs_min_x+maxr_x;
    sec_min=sec_minr;
    sec_min_x=sec_minr_x+abs_min_x+maxr_x;
else
    [sec_minl, sec_minl_x]=min(y(1:maxl_x));
    maxg=maxl;
    max_x=maxl_x;
    sec_min=sec_minl;
    sec_min_x=sec_minl_x;
end
heights(i)=maxg-(abs_min+sec_min)/2

end
%%
if length(heights) < 3
    heights=[heights NaN(1,3-length(heights))];
end
heights(4)=mean(heights(1:3),'omitnan');
heights(5)=std(heights(1:3),'omitnan');
dlmwrite('result.csv',heights,'delimiter','\t','-append');

```

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