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Differential and Isomer-Specific Modulation of Vitamin A Transport and the Catalytic Activities of the RBP Receptor by Retinoids

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Abstract Retinoids are vitamin A derivatives with diverse biological functions. Both natural and artificial retinoids have been used as therapeutic reagents to treat human diseases, but not all retinoid actions are understood mechanistically. Plasma retinol binding protein (RBP) is the principal and specific carrier of vitamin A in the blood. STRA6 is the membrane receptor for RBP that mediates cellular vitamin A uptake. The effects of retinoids or related compounds on the receptor's vitamin A uptake activity and its catalytic activities are not well understood. In this study, we dissected the membrane receptor-mediated vitamin A uptake mechanism using various retinoids. We show that a subset of retinoids strongly stimulates STRA6-mediated vitamin A release from holo-RBP. STRA6 also catalyzes the exchange of retinol in RBP with certain retinoids. The effect of retinoids on STRA6 is highly isomer-specific. This study provides unique insights into the RBP receptor's mechanism and reveals that the vitamin A transport machinery can be a target of retinoid-based drugs.

Keywords Membrane receptor · Membrane transport · Vitamin A transport · Retinoid

Riki Kawaguchi and Ming Zhong have contributed equally to this study.

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Introduction

Retinoids are a group of organic molecules derived from vitamin A and include both natural and artificial compounds. In addition to the critical role of vitamin A aldehyde in vision (Crouch et al. 1996; Dowling 1966; Kefalov 2012; Travis et al. 2007; Wald 1968), most of the biological effects of vitamin A are mediated by retinoic acids, which are ligands for nuclear hormone receptors to regulate gene transcription (Chambon 1996; Evans 1994; Napoli 1996; Ross 1993). Other retinoids may also have distinct biological activities. For example, retinol, but not retinoic acid, can reverse the degeneration of germinal epithelium in testis caused by vitamin A deficiency (Griswold et al. 1989; Howell et al. 1963). Retinol, but not retinoic acid, regulates BMP4 expression in male germ line cells (Baleato et al. 2005). An imbalance in retinoid homeostasis is associated with a variety of human diseases including visual disorders (Travis et al. 2007), cancer (Love and Gudas 1994; Niles 2004; Verma 2003), infectious diseases (Ross 1992; Sommer 1993; Stephensen 2001), neurological disorders (Drager 2006; Maden 2007), diabetes (Basu and Basualdo 1997), teratogenicity (Nau et al. 1994) and skin diseases (Chivot 2005; Orfanos et al. 1997; Zouboulis 2001).

Perhaps the earliest retinoid-based therapy was the ancient Egyptians' use of fresh liver to cure night blindness. The biochemical basis of this therapy was not known until thousands of years later when the active ingredient was identified as vitamin A and vitamin A was revealed as the chromophore for photoreceptor cells in vision (Dowling 1966). In modern times, natural and artificial retinoids have been used to treat human diseases, especially in oncology and dermatology. For example, a combination of retinoic acid and arsenic trioxide offers a highly effective treatment

for acute promyelocytic leukemia (Zhou et al. 2007). This therapy was one of the most successful cures of a special type of cancer. Retinoic acid (Acutane) is also an effective drug to treat acne; but its mechanism of action in acne treatment is still not understood, and it has many side effects including teratogenicity and depression.

The principal and specific carrier of vitamin A in the blood is plasma retinol binding protein (RBP) (Blomhoff et al. 1990; Goodman 1984; Newcomer and Ong 2000; Quadro et al. 2003; Rask et al. 1980; Tanumihardjo 2004; Zanotti and Berni 2004), which is essential to mobilize the vitamin A stored in the liver. RBP delivers vitamin A to target organs by binding to its specific cell-surface receptor, STRA6 (Kawaguchi et al. 2007). Loss of STRA6 leads to severe pathological phenotypes in multiple human organs including the eye, brain, lung and heart (Golzio et al. 2007; Pasutto et al. 2007). Studies in animal models demonstrated that loss of STRA6 leads to highly suppressed vitamin A uptake in zebrafish (Isken et al. 2008) and in mice (Ruiz et al. 2012). STRA6 is responsible for 95 % of retinoid uptake for vision under vitamin A–sufficient conditions (Ruiz et al. 2012). What is responsible for the STRA6-independent 5 % that partially compensates for the loss of STRA6? The small intestine-secreted lipoprotein particle that contains retinyl ester is the predominant RBP-independent pathway to transport vitamin A in the blood and is known to partially compensate the RBP pathway under vitamin A-sufficient or -excessive conditions (Blomhoff et al. 1990; D'Ambrosio et al. 2011; Smith and Goodman 1976; Zhong et al. 2012). Under vitamin A-deficient conditions that mimic natural environments, RBP knockout is embryonic lethal due to the inability of the retinyl ester pathway to mobilize stored vitamin A in the liver (Quadro et al. 2004, 2005).

STRA6 represents a new type of cell-surface receptor that has nine transmembrane domains (Kawaguchi et al. 2008b). An essential RBP binding domain has been identified between transmembrane domains VI and VII (Kawaguchi et al. 2008a). STRA6-mediated vitamin A uptake employs a membrane transport mechanism distinct from known mechanisms such as active transport (which depends on cellular energy), channels and facilitated transport (which depends on the electrochemical gradient of the free substrate). Unlike soluble substrates, STRA6s substrate, vitamin A, is only provided one at a time through RBP and is not free but bound with high affinity to RBP. STRA6 functions as a receptor that not only binds to both holo-RBP and apo-RBP (Kawaguchi et al. 2012) but also catalyzes retinol release from holo-RBP (Kawaguchi et al. 2011) and retinol loading into apo-RBP (Isken et al. 2008; Kawaguchi et al. 2011). STRA6-catalyzed retinol release activity is largely responsible for its ability to take up vitamin A from RBP, which remains outside of the cell (Kawaguchi et al. 2011). The ability of STRA6-mediated retinol loading to counteract STRA6's retinol release activity is responsible for STRA6's coupling to intracellular proteins involved in retinoid storage (Kawaguchi et al. 2011).

Although a few studies have investigated the interaction of retinoids with RBP to gain an understanding of RBP's mechanism (Berni et al. 1993; Goodman and Raz 1972; Horwitz and Heller 1973, 1974), it was not previously feasible to study the specific interaction between retinoids and the RBP receptor before its identification due to the complications from endogenous retinoid binding proteins and related enzymes. In this study, we used several complementary techniques to study whether or how distinct retinoids affect the transport activity and catalytic activities of the RBP receptor. Using both natural and artificial retinoids, this study revealed the differential and isomer-specific interactions between retinoids and the vitamin A uptake mechanism mediated by the RBP receptor and offers unique insights into the receptor's substrate uptake mechanism.

Materials and Methods

Production and Purification of Holo-RBP, Apo-RBP and ^{3}H-retinol/RBP

Holo-RBP was produced, refolded in the presence of retinol and purified as described (Kawaguchi et al. 2007). HPLC was used to purify holo-RBP 100 % loaded with retinol. Apo-RBP was prepared by extracting retinol from holo-RBP as described (Heller and Horwitz 1973). For ^{3}H-retinol/RBP, ^{3}H-retinol (PerkinElmer, Waltham, MA) was incubated with apo-RBP (with 6XHis tag at the N terminus) overnight at 4 °C. ^{3}H-retinol/RBP was purified using Ni-NTA resin (Qiagen, Hilden, Germany) and used immediately for cellular uptake assays.

Assay for Cellular Uptake of ^{3}H-retinol from ^{3}H-retinol/RBP

Cells grown in regular medium containing 10 % serum were washed once with Hank's balanced salt solution (HBSS) and grown in serum-free medium (SFM) overnight before the assay. Overnight incubation in SFM allowed the dissociation of RBP in the serum as this association can block the binding of ^{3}H-retinol/RBP. On the day of the assay, fresh SFM was added to the cells before the addition of 50 nM retinoids (7.7 times the concentration of ^{3}H-retinol/RBP). All experiments involving retinoid isomers were done under red dim light to avoid light-induced isomerization. ^{3}H-retinol/RBP (6.5 nM) was then added. After a 1-h incubation at 37 °C in the dark, cells were washed with HBSS and solubilized in 1 % Triton X-100 in

PBS. The ^{3}H-retinol remaining in the cells was measured with a scintillation counter.

Assay of STRA6-Mediated Retinoid Exchange

Cell growth and retinoid addition were done similarly as described above for the radioactive assay. Five minutes after retinoid addition to cells, His-tagged-holo-RBP was added to the cells. After incubation at 37 °C in the dark for 20 min, supernatant was transferred to a new tube containing Ni-NTA. After purification of His-tagged RBP under red dim light, retinoid was extracted from purified RBP by adding an equal volume of ethanol containing 1 mM BHT. After mixing with a vortex for 5 min, hexane was added and vortexing continued for 5 min. After centrifugation at 5,000×*g* for 5 min, the organic phase was transferred to a new tube prewashed with hexane. Extraction of hexane was repeated twice, and extracts were combined. Hexane was then evaporated under vacuum.

LC/MS/MS Analysis of Retinoids

Hexane-extracted retinoid was resuspended in methanol containing 1 % acetic acid. Retinoid was then loaded onto nLC with Easy-Column (C18, 10 cm, 3 μm; Thermo Fisher, Waltham, MA) with 80 % methanol containing 1 % acetic acid, separated in nLC in isocratic mode with methanol containing 1 % acetic acid and analyzed with a Thermo LTQ Velos mass spectrometer. Precursor ions were monitored from 250 to 550 in M/Z with automatic gain control (AGC) target of 30,000 and 10 ms of maximum injection time (IT). Detection and quantification of retinoid were done in the MS2 mode, and the monitored ions were as follows: fenretinide (392 to 283) and all-*trans*, 9-*cis*- and 13-*cis*-retinoic acids (301 to 205) (Supplemental Fig. 1). In the Xcalibur program (Thermo Fisher, Waltham, MA), a parent mass list was created for all retinoid species, and these ions were constantly monitored throughout the gradient run. AGC and IT for MS/MS were set at 10,000 and 100 ms, respectively. Collision-induced dissociation was done at 30 % energy. Smoothing was applied to the MS/MS signal by the boxcar method for every seventh data point. Peak detection and calculation of signal area were done in the XCalibur program using default settings (baseline window = 40, area noise factor = 5 and peak noise factor = 10).

Real-Time Analysis of STRA6-Catalyzed Retinol Loading and Retinol Release

Retinol fluorescence was measured using POLARstar Omega (BMG Labtech, Cary, NC) with the excitation filter 320ex and the emission filter 460-10 as previously described (Kawaguchi et al. 2013). Briefly, the Microfluor-2 plate (Thermo Fisher) was coated with Blocker Casein (Pierce, Rockford, IL) overnight at 4 °C and washed with PBS to prevent nonspecific sticking of holo-RBP to the plastic wall. Membranes were resuspended in PBS and mixed with various proteins, then transferred to the plate for fluorescent measurement. The signal from each time point is the average of 10 measurements. Samples were shaken for 10 s at 500 rpm using double-orbital shaking before each measurement. Background signals before the addition of retinol or holo-RBP at 0 min were subtracted from the final fluorescence signals at each time point.

Real-Time Analysis of STRA6-Catalyzed Retinol Transport from Holo-RBP to EGFP-CRBP-I Using Fluorescence Resonance Transfer

Retinol and EGFP have been established as a fluorescence resonance transfer (FRET) pair previously (Kawaguchi et al. 2011). Retinoids were added to membranes under red dim light. Reactions were initiated by adding holo-RBP to EGFP-CRBP-I premixed with membranes. Retinol-EGFP FRET was measured with the excitation filter 320ex and the emission filters 460-10 and 510-10 using simultaneous dual-emission optics in POLARstar Omega. The signal from each time point was the average of 10 measurements. The background signal of each reaction was measured before holo-RBP was added at 0 min. Retinal-EGFP FRET was calculated using the equation $(510_t - 510_b)/(460_t - 460_b)$, where 510_t, 510_b, 460_t and 460_b represent emissions at 510 nm after initiation of the reaction (t = time point), at 510 nm before holo-RBP is added (b = background), at 460 nm after initiation of the reaction and at 460 nm before holo-RBP is added, respectively.

Statistical Analysis

Raw data were initially processed in Excel and then imported to RStudio (version 0.97.318, www.rstudio.com). One-way ANOVA and post-hoc analysis were performed using the Multcomp package (http://cran.r-project.org/web/packages/multcomp/index.html).

Results

Differential Effects of Retinoids on STRA6-Catalyzed Retinol Release from Holo-RBP

A key activity of STRA6 is to catalyze retinol release from holo-RBP (Kawaguchi et al. 2011). This activity is responsible for endocytosis-independent uptake of vitamin A from holo-RBP. We tested the effects of a few representative retinoids on this key activity of STRA6, including fenretinide, all-*trans*-retinal, 9-*cis*-retinal, all-*trans*-retinoic acid, 9-*cis*-

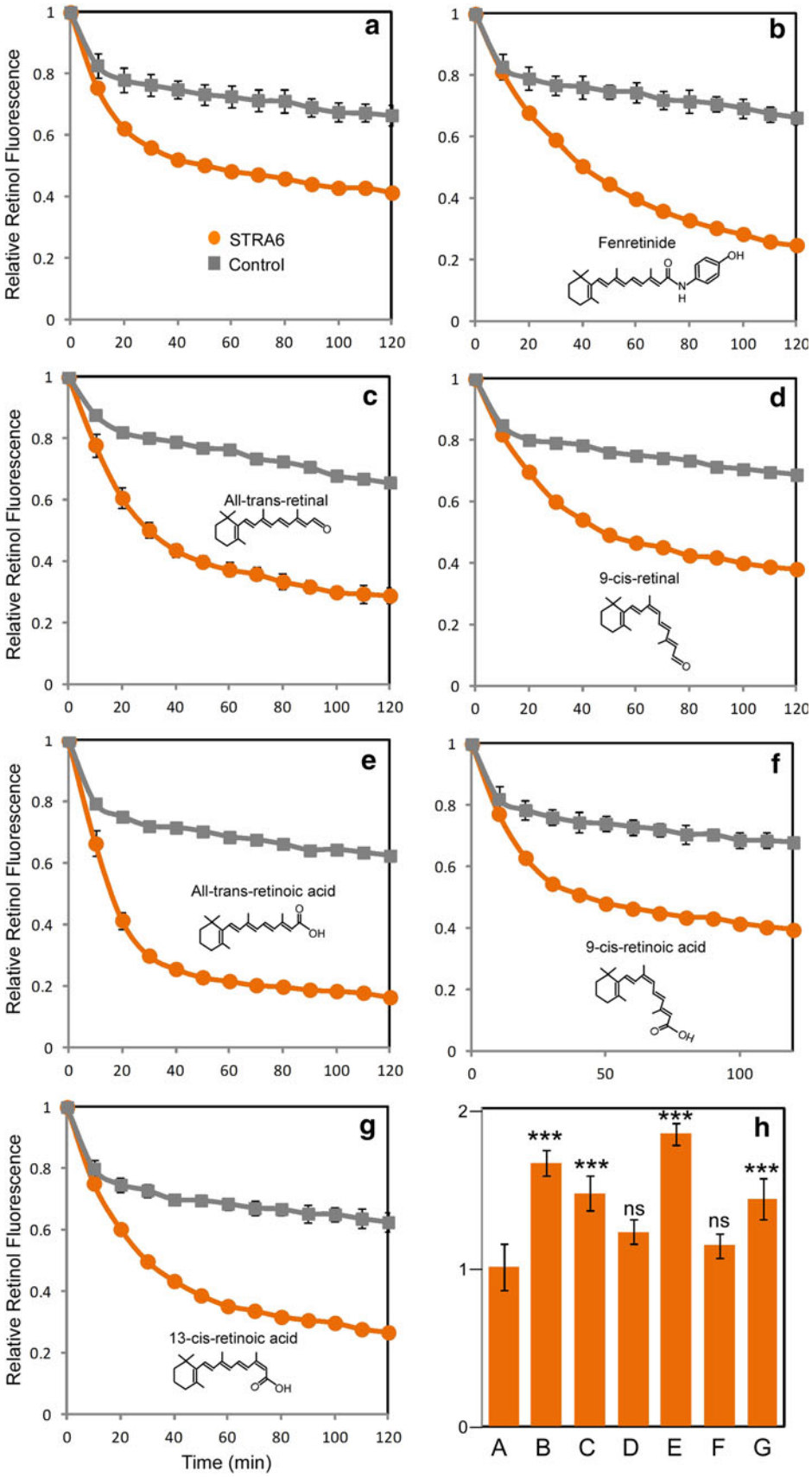


Fig. 1 Isomer-specific effect of retinoids on STRA6-mediated retinol release from holo-RBP. **a** Time course of STRA6-catalyzed retinol release from 1 μM of holo-RBP without retinoid addition. **b–g** Effects of 10 μM of fenretinide, all-*trans*-retinal, 9-*cis*-retinal, all-*trans*-retinoic acid, 9-*cis*-retinoic acid and 13-*cis*-retinoic acid, respectively. Retinoid was added to both the STRA6 reaction and the control reaction without STRA6. For **a–g**, retinol fluorescence at time 0 is defined as 1. **a–g** STRA6-dependent retinol release activity without retinoid is defined as 1. **h** Statistical analysis of retinol release activity at 120 min for experiments. Retinol release activity without retinoid addition is defined as 1. Statistical significance is shown as $***p < 0.001$ or ns (not significant)

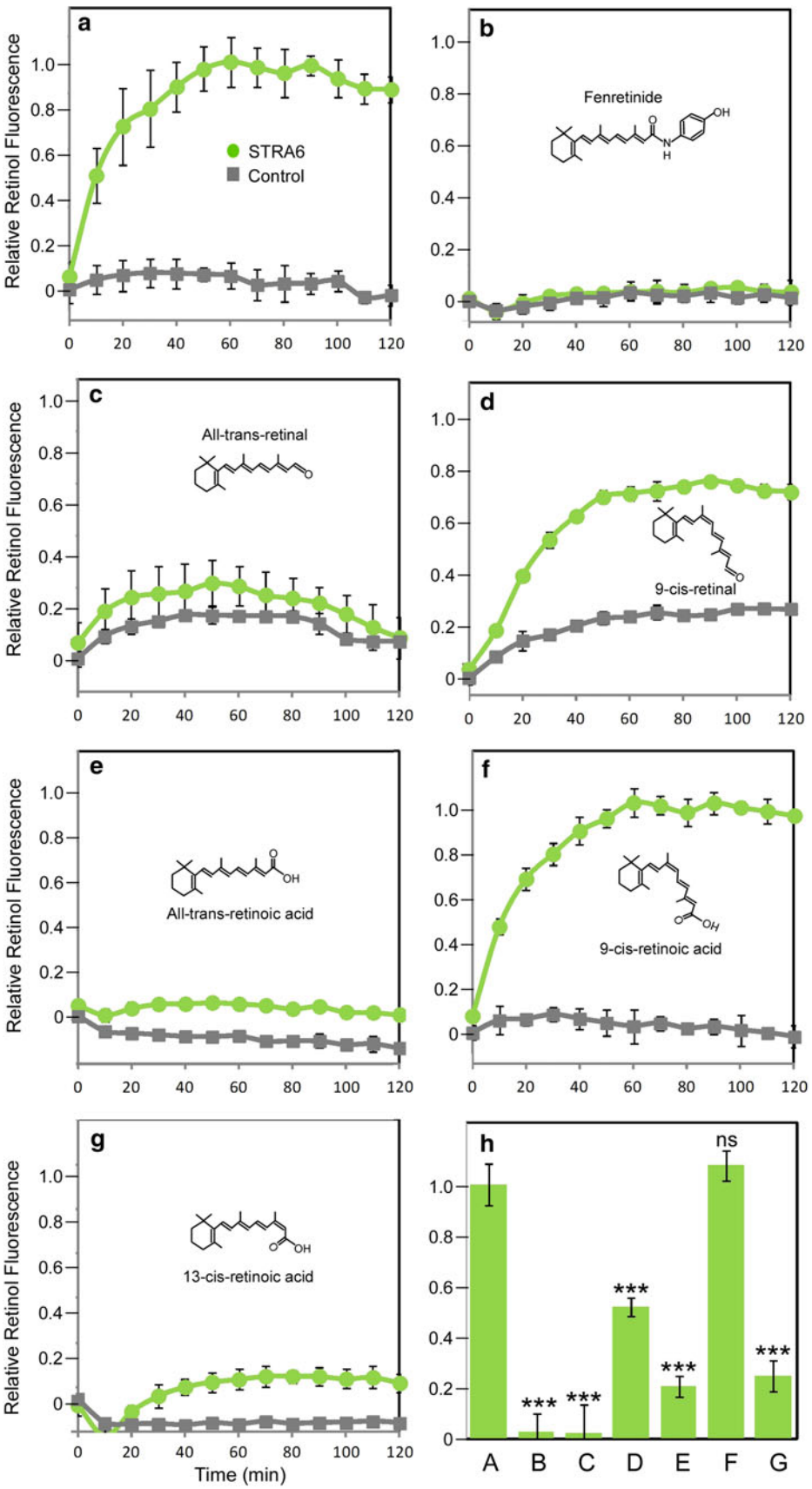


Fig. 2 Isomer-specific effect of retinoids in inhibiting STRA6-catalyzed retinol loading into apo-RBP. **a** Time course of STRA6-catalyzed retinol loading of apo-RBP without retinoid addition (1 μM retinol and 1 μM apo-RBP were added at time 0). **b–g** Effects of 20 μM of fenretinide, all-*trans*-retinal, 9-*cis*-retinal, all-*trans*-retinoic acid, 9-*cis*-retinoic acid and 13-*cis*-retinoic acid, respectively. Retinoid was added to both the STRA6 reaction and the control reaction without STRA6. For **a–g**, the highest retinol loading activity of STRA6 without exogenous retinoid is defined as 1. **a–g** STRA6-dependent retinol loading activity without retinoid is defined as 1. **h** Statistical analysis of STRA6-dependent retinol loading activity at 120 min for experiments. Retinol loading activity without retinoid addition is defined as 1. Statistical significance is shown as $***p < 0.001$ or ns (not significant)

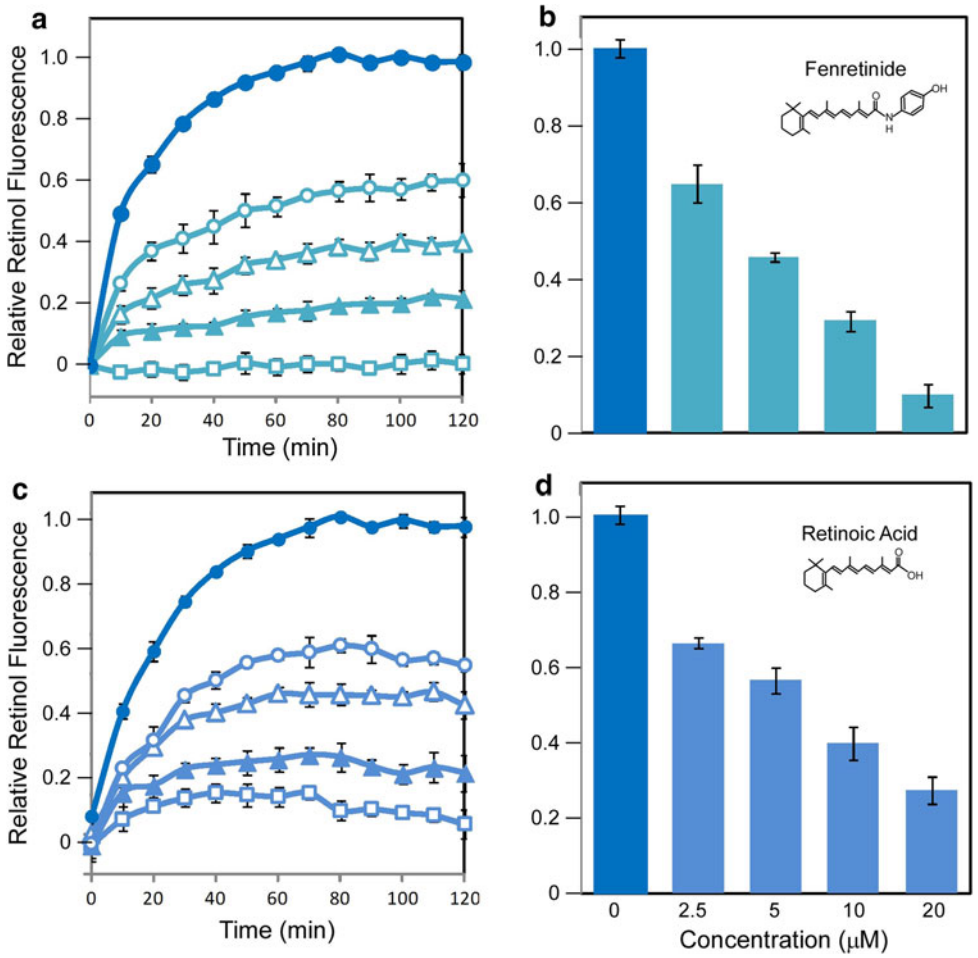


Fig. 3 Dose dependence of the effect of retinoid on STRA6-catalyzed retinol loading. **a** Real-time analysis of STRA6-catalyzed retinol (1 μM) loading into apo-RBP (1 μM) showing the suppression by different concentrations of fenretinide (*open circle* 2.5 μM, *open triangle* 5 μM, *closed triangle* 10 μM, *open square* 20 μM). The highest retinol fluorescence without adding fenretinide is defined as 1. **b** Comparison of the suppression effect of different fenretinide concentrations at 120 min of STRA6-catalyzed retinol loading into apo-RBP. Retinol loading activity without fenretinide addition is defined as 1. **c** Real-time analysis of STRA6-catalyzed retinol (1 μM) loading into apo-RBP (1 μM) showing the suppression by different concentrations of retinoic acid (*open circle* 2.5 μM, *open triangle* 5 μM, *closed triangle* 10 μM, *open square* 20 μM). The highest retinol fluorescence without adding retinoic acid is defined as 1. **d** Comparison of the suppression effect of different retinoic acid concentrations at 120 min of STRA6-catalyzed retinol loading into apo-RBP. Retinol loading activity without retinoic acid addition is defined as 1

retinoic acid and 13-*cis*-retinoic acid. We found that these retinoids differentially stimulate STRA6's retinol release activity. Although all these retinoids are known to bind to RBP (Berni et al. 1993; Goodman and Raz 1972; Horwitz and Heller 1973, 1974), 9-*cis*-retinal and 9-*cis*-retinoic acid are much less effective than all-*trans*-retinal, all-*trans*-retinoic acid, 13-*cis*-retinoic acid and fenretinide (Fig. 1).

Differential Effects of Retinoids on STRA6-Catalyzed Retinol Loading into Apo-RBP

A second key activity of STRA6 is to catalyze retinol loading into apo-RBP (Kawaguchi et al. 2011). This activity counteracts STRA6-catalyzed retinol release and is responsible for the coupling of STRA6-mediated vitamin A uptake to intracellular proteins involved in retinoid storage. We observed the reciprocal effects of these retinoids on STRA6-catalyzed retinol release activities. The retinoids that strongly stimulate STRA6-mediated vitamin A uptake strongly inhibit STRA6-mediated retinol loading. In this case, fenretinide, all-*trans*-retinoic acid, all-*trans*-retinal and 13-*cis*-retinoic acid are much more effective than 9-*cis*-retinoic acid and 9-*cis*-retinal in inhibiting STRA6-catalzyed retinol loading (Fig. 2). We also demonstrated that the effect of retinoids is dose-dependent (Fig. 3). These results suggest that retinoids stimulate STRA6-

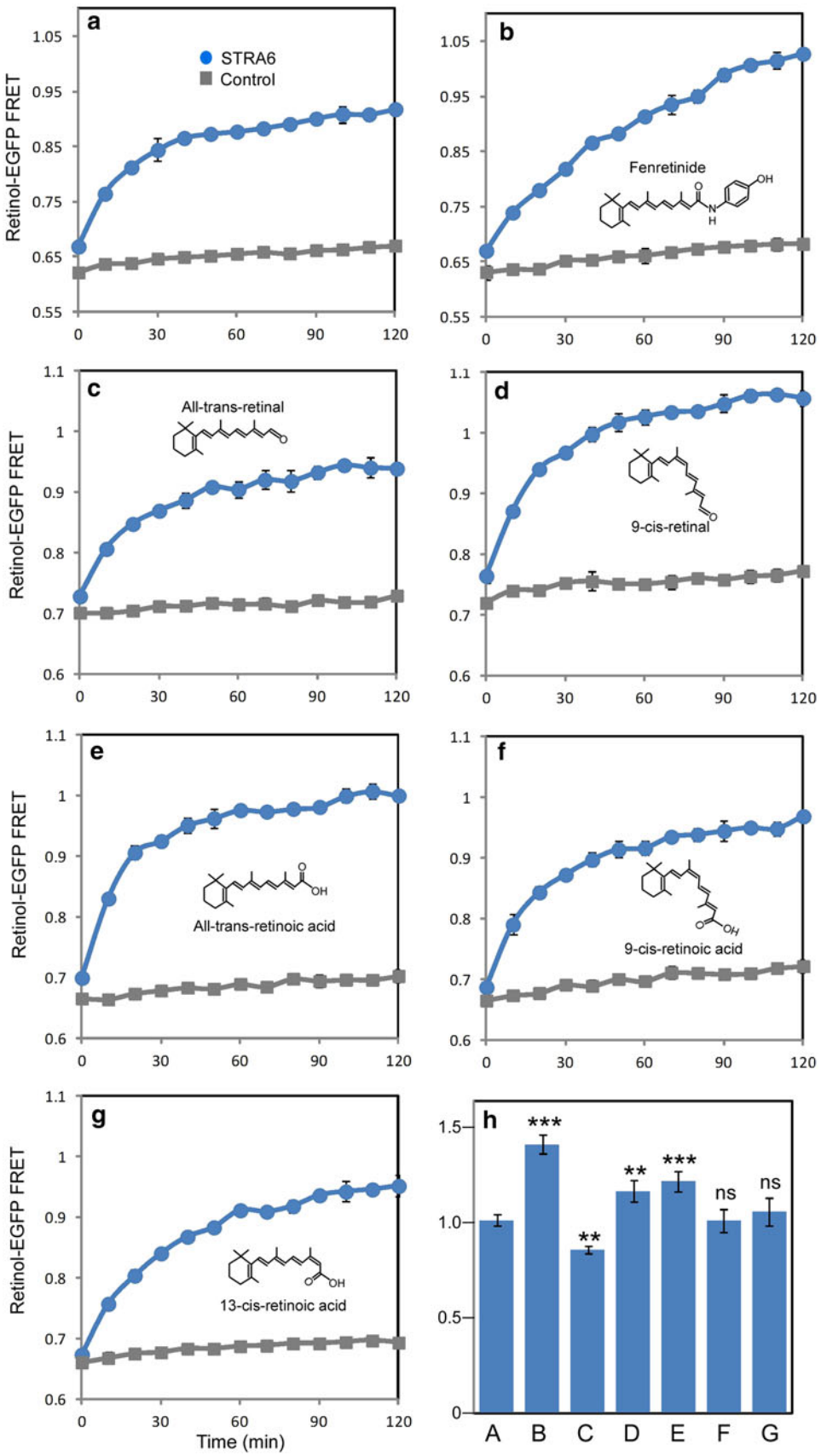


Fig. 4 Isomer-specific effect of retinoids on STRA6-catalyzed retinol transport from holo-RBP to EGFP-CRBP-I as measured by retinol-EGFP FRET. **a** Time course of STRA6-catalyzed retinol transport from holo-RBP to EGFP-CRBP-I (2 μM holo-RBP and 1 μM EGFP-CRBP-I were added at time 0). **b–g** Effects of 20 μM of fenretinide, all-*trans*-retinal, 9-*cis*-retinal, all-*trans*-retinoic acid, 9-*cis*-retinoic acid and 13-*cis*-retinoic acid, respectively. Retinoid was added to both the STRA6 reaction and the control reaction without STRA6. For **a–g**, retinol fluorescence at time 0 is defined as 1. STRA6-dependent retinol transport activity without retinoid is defined as 1. **h** Statistical analysis of retinol release activity at 120 min for experiments (**a–g**). Retinol transport activity without retinoid addition is defined as 1. Statistical significance is shown as $^{**}p < 0.01$, $^{***}p < 0.001$ or ns (not significant)

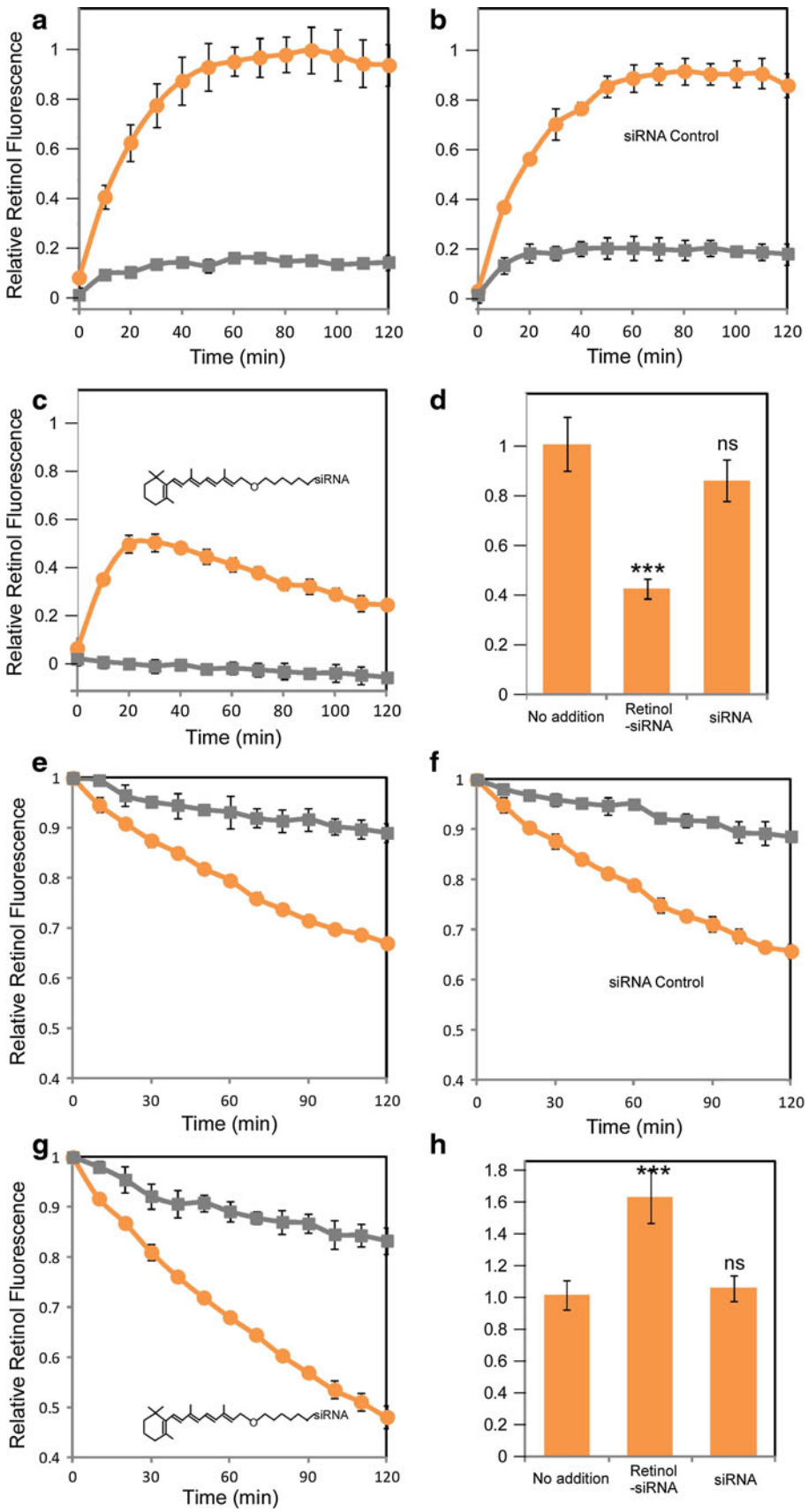
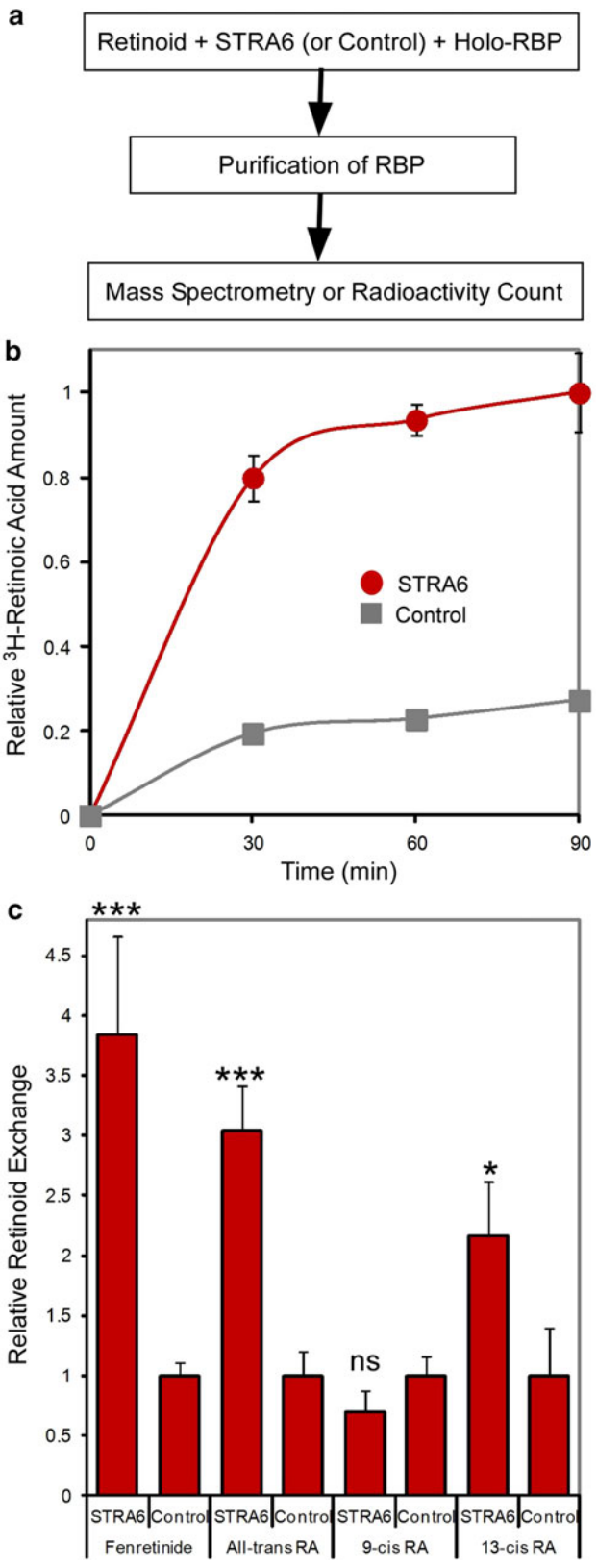


Fig. 5 Effects of retinol-siRNA conjugate on STRA6-mediated retinol loading and STRA6-mediated retinol release activities. **a–d** Retinol-siRNA inhibits STRA6-catalyzed retinol (1 μM) loading into apo-RBP (1 μM). **b**, **c** STRA6-mediated retinol loading into apo-RBP in the presence of siRNA (20 μM) and retinol-siRNA (20 μM), respectively, compared to no addition control in (**a**). For **a–c**, the highest retinol loading activity in **a** is defined as 1. **d** Quantitation of STRA6's retinol loading activity in **a–c**. Retinol loading activity without addition of retinol-siRNA or siRNA is defined as 1. **e–h** Retinol-siRNA stimulates STRA6-catalyzed retinol release from holo-RBP (1 μM). **f**, **g** STRA6-mediated retinol release from holo-RBP in the presence of siRNA (20 μM) and retinol-siRNA (20 μM), respectively, compared to no addition control in **e**. For **e–g**, retinol fluorescence at time 0 is defined as 1. **h** Quantitation of STRA6's retinol release activity in **e–g**. Retinol loading activity without addition of retinol-siRNA or siRNA is defined as 1. Statistical significance is shown as $***p < 0.001$ or ns (not significant)



◀ **Fig. 6** STRA6 catalyzes retinoid exchange with retinol in holo-RBP. **a** Brief summary of the experimental design. **b** Radioactive assay showing that STRA6 catalyzes the exchange of ^{3}H-retinoic acid with retinol in holo-RBP. The highest exchange activity is defined as 1. **c** Mass spectrometric analysis of retinoid exchange into holo-RBP by STRA6. Retinoid exchange activity of the control reaction without STRA6 is defined as 1. Statistical significance is shown as $*p < 0.05$, $***p < 0.001$ or ns (not significant)

mediated retinol release by inhibiting STRA6-mediated retinol loading, which counteracts the retinol release activity.

Differential Effects of Retinoids on STRA6-Catalyzed Retinol Transport from Holo-RBP to CRBP-I

We next analyzed these retinoids on STRA6-catalyzed retinol transport from holo-RBP to CRBP-I in real time by monitoring retinol-EGFP FRET (Fig. 4). Interestingly, the effects of retinoids on this activity are similar but not identical to their effects on STRA6's retinol release activity. The likely explanation is that this assay is complicated by the interaction of retinoids with CRBP-I. This experiment demonstrates the importance of isolating the RBP receptor in studying its specific interaction with retinoids.

STRA6 Catalyzes the Loading of Retinol-Sirna Conjugate into Apo-RBP

The previous experiments showed that the configuration of the double bonds in retinoids can affect their effects on STRA6-mediated activities. We next tested a retinol conjugate with siRNA, which adds a long soluble tail to retinol. Like all other all-*trans*-retinoids, retinol-siRNA effectively stimulates STRA6-mediated retinol release from holo-RBP and inhibits STRA6-mediated retinol loading into apo-RBP (Fig. 5). These effects suggest that the long soluble siRNA tail of the retinol-siRNA conjugate does not interfere with its ability to influence STRA6's activities.

STRA6 Catalyzes Isomer-Specific Exchange of Retinoid with Retinol in Holo-RBP

Since retinoids can inhibit STRA6-mediated retinol loading into apo-RBP, it is likely that the inhibition is caused by STRA6's loading of retinoids other than retinol into apo-RBP. The competition between exogenous retinoids with retinol to be loaded into apo-RBP is at least partly responsible for the inhibition. If this hypothesis is true, retinoids should exchange with retinol in the presence of STRA6, which can catalyze retinol release from holo-RBP and retinoid loading into apo-RBP. To test this hypothesis, we incubated various retinoids with holo-RBP in the presence or absence of STRA6 and then purified RBP to analyze if exogenous retinoid is bound (Fig. 6).

We first tested all-*trans*-retinoic acid, which has a radioactive form available, and found that STRA6 catalyzes the exchange of unlabeled retinol in holo-RBP with ^{3}H-retinoic acid (Fig. 6b). We also developed a mass spectrometry–based technique to quantify fenretinide and different isomers of retinoic acid in purified RBP (Supplemental Fig. 1). Using this technique, we demonstrated isomer-specific exchange of retinoid with retinol in holo-RBP. Consistent with previous experiments that analyzed STRA6-catalyzed retinol loading and STRA6-catalyzed retinol release, STRA6 effectively promotes the exchange of fenretinide, all-*trans*-retinoic acid and 13-*cis*-retinoic acid, but not 9-*cis*-retinoic acid, with retinol in RBP (Fig. 6c).

Differential Effects of Retinoids on STRA6-Mediated Cellular Vitamin A Uptake

We next tested these retinoids in the cellular vitamin A uptake assay using ^{3}H-retinol/RBP (Fig. 7). Interestingly, none of the retinoids had a significant effect on retinol release on holo-RBP in the absence of STRA6, but STRA6 enabled strong retinol release abilities by a subset of these retinoids (Fig. 7). Consistent with previous experiments, the effects of retinoids are highly isomer-specific. Both 9-*cis*-isomers were much less effective than their all-*trans* counterparts. However, the biggest surprise is that retinol-siRNA had no effect on STRA6-mediated ^{3}H-retinol uptake from ^{3}H-retinol/RBP, although retinol-siRNA was very effective in stimulating STRA6-catalyzed retinol release from holo-RBP and STRA6-catalzyed retinol loading into apo-RBP in membrane-based assays (Fig. 5). This behavior is in contrast to other all-*trans*-retinoids, which stimulate both STRA6-catalyzed retinol release in a membrane-based assay and ^{3}H-retinol uptake from ^{3}H-retinol/RBP in the live cell assay. This surprising result will be discussed further in "Discussion" section.

Comparative Study of all-*trans*-Retinol and 9-*cis*-Retinol

All previous experiments were performed using retinoids that do not have the alcohol group. To specifically dissect the influence of the double bond configuration on STRA6's catalytic activities, we did a comparative study of all-*trans*-retinol and 9-*cis*-retinol (Fig. 8). 9-*cis*-Retinol differs from all-*trans*-retinol only in its double bond configuration. Indeed, 9-*cis*-retinol behaves very differently from all-*trans*-retinol. Compared to all-*trans*-retinol, 9-*cis*-retinol is a poor substrate for STRA6 and has little effect on STRA6-mediated release of all-*trans*-retinol from holo-RBP or STRA6-mediated loading of all-*trans*-retinol into apo-RBP. Consistent with previous experiments using other retinoids, this comparative study of all-*trans*-retinol and 9-*cis*-retinol demonstrates that the configuration of the double bond has a substantial impact on the effects of retinoids on STRA6's activities.

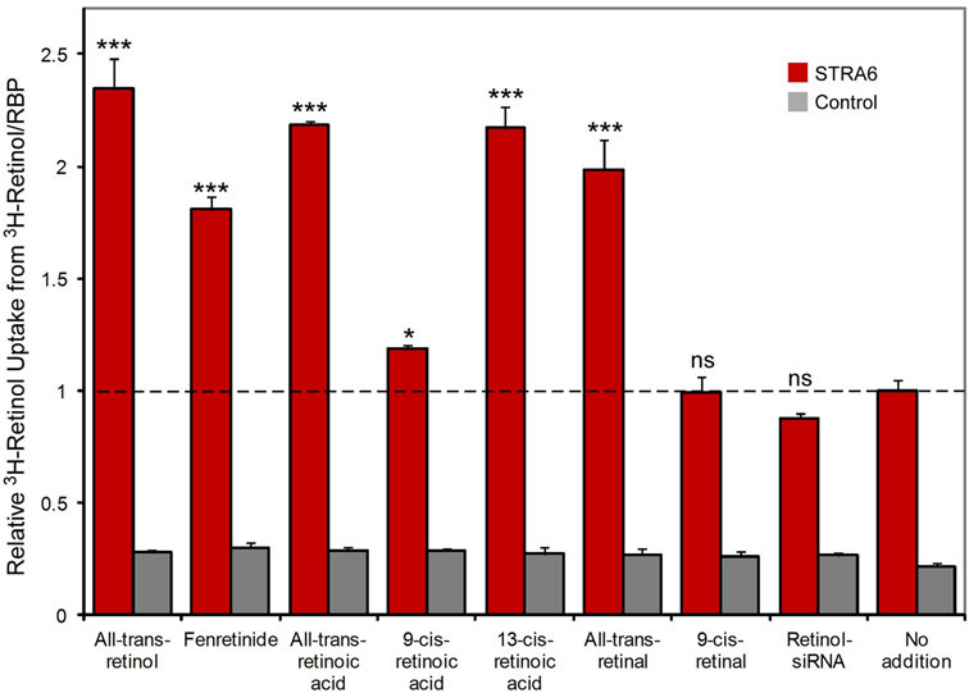


Fig. 7 Assays of ^{3}H-retinol uptake from ^{3}H-retinol/RBP showing that STRA6 enables a subset of retinoids to strongly stimulate retinol release from holo-RBP. STRA6's retinol uptake activity from holo-RBP without exogenous retinoid is defined as 1. Statistical significance is shown as $*p < 0.05$, $***p < 0.001$ or ns (not significant)

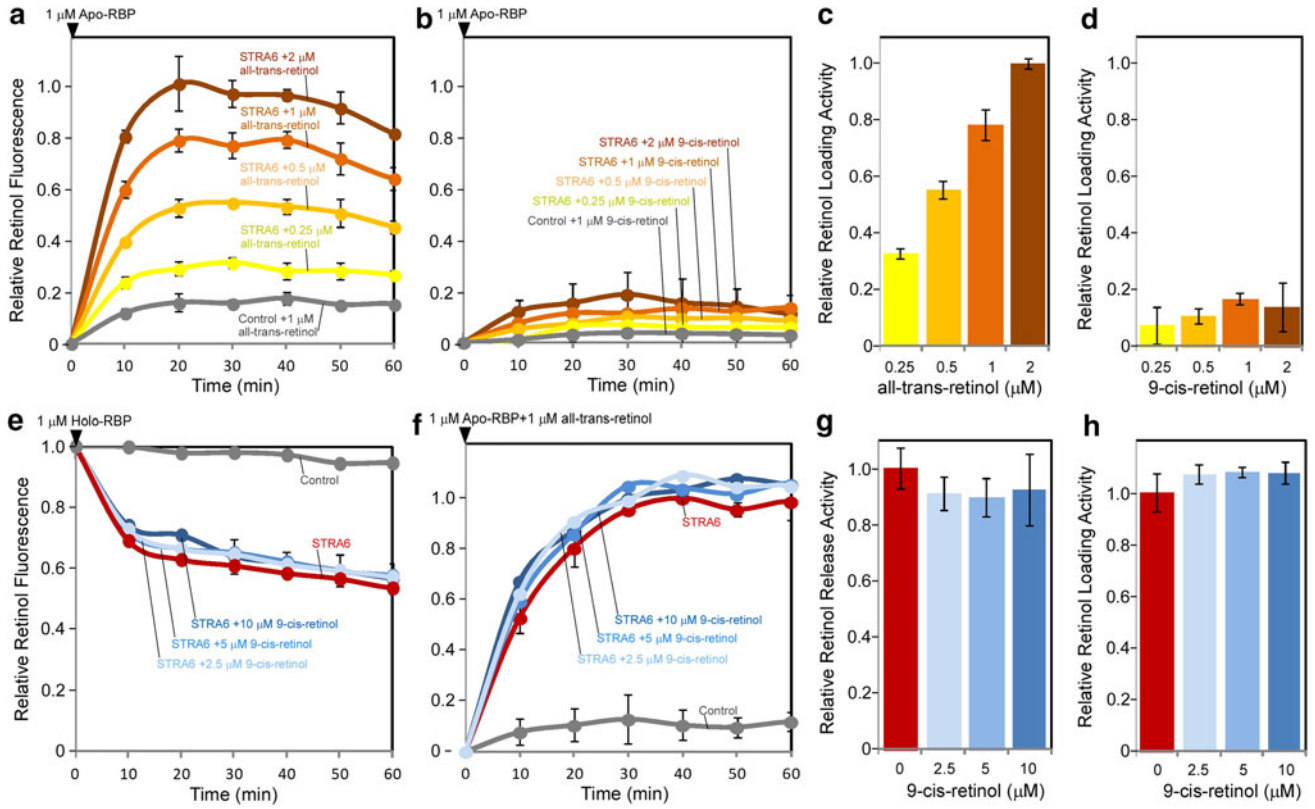


Fig. 8 Comparative study of all-*trans*-retinol and 9-*cis*-retinol. **a**, **b** Comparison of STRA6-catalyzed loading of all-*trans*-retinol (**a**) and 9-*cis*-retinol (**b**) into apo-RBP. Apo-RBP (1 μM) and different concentrations of retinol were added at time 0. The highest signal for all-*trans*-retinol is defined as 1. **c**, **d** Relative STRA6-dependent retinol loading activities at 60 min for experiments for **a** and **b**, respectively. The highest signal for all-*trans*-retinol is defined as 1. **e** Effects of different concentrations of 9-*cis*-retinol on STRA6-catalyzed retinol release from holo-RBP (1 μM). Retinol fluorescence at time 0 is defined as 1. **f** Effects of different concentrations of 9-*cis*-retinol on STRA6-catalyzed loading of all-*trans*-retinol (1 μM) into apo-RBP (1 μM). The highest STRA6-dependent signal without 9-*cis*-retinol addition is defined as 1. **g**, **h** The effects of 9-*cis*-retinol at 60 min for experiments **a** and **b**, respectively. The signal without 9-*cis*-retinol addition is defined as 1 (*red bars*)

Discussion

The best-known targets of retinoids are nuclear hormone receptors, but whether the RBP receptor STRA6 is a target of retinoids is not well understood. Understanding the molecular mechanism of small molecule effects on the RBP receptor will help to not only reveal the receptor's molecular mechanism but also develop better strategies to increase or decrease tissue retinoid levels as a strategy to treat human diseases. Using a combination of complementary radioactivity-based, mass spectrometry-based and real-time fluorescence-based techniques, we specifically studied the effects of retinoids on STRA6 and showed that it is also a target of certain retinoids.

These experiments provide several unique insights into STRA6's vitamin A uptake mechanism (Fig. 9). First, certain retinoids can have profound effects on STRA6's catalytic activities. For example, fenretinide can completely suppress STRA6's vitamin A loading activity into RBP and strongly stimulate STRA6-catalyzed retinol release from holo-RBP.

Second, all retinoids tested that enhance STRA6-mediated vitamin A uptake influence STRA6's activities by inhibiting STRA6-catalyzed retinol loading. Because STRA6's retinol loading activity counteracts its retinol release activity, inhibiting retinol loading results in stimulation of STRA6-catalyzed retinol release. In other words, these retinoids stimulate retinol release by causing a short circuit in the cycle of STRA6-catalyzed retinol release and retinol loading.

Third, effects of retinoids are highly isomer-specific. Generally, all-*trans* isomers have much more potent effects on STRA6 than 9-*cis* isomers. Since 9-*cis*-retinoid does bind RBP to form a 1:1 complex (Heller and Horwitz 1973; Horwitz and Heller 1973), the inability of 9-*cis*-retinoid to stimulate STRA6-mediated vitamin A uptake is likely due to its inability to pass through STRA6 effectively (Fig. 9). The isomer-specific effects of retinoid's action demonstrate the high

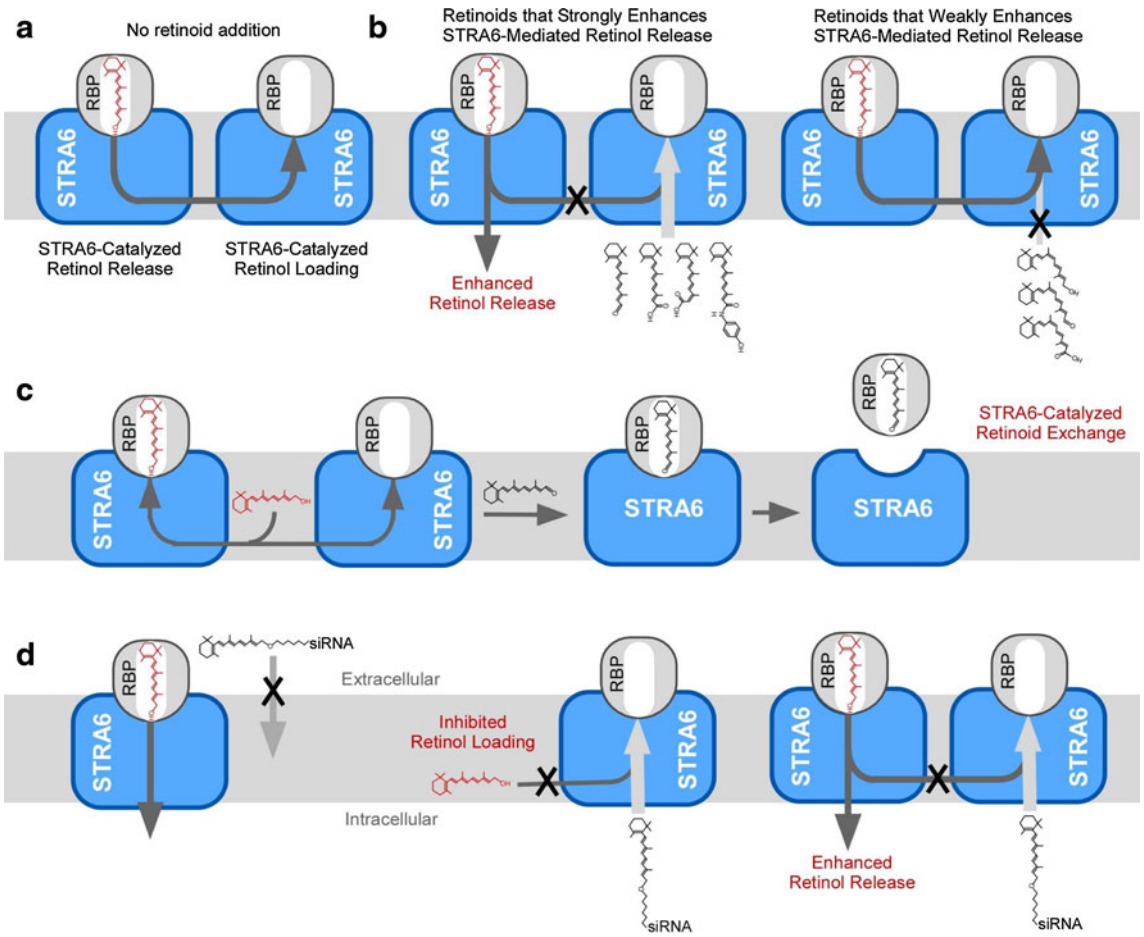


Fig. 9 Schematic diagrams of the effects of retinoids on STRA6 activities. **a** STRA6 catalyzes retinol loading into apo-RBP and retinol release from holo-RBP. **b** Fenretinide, all-*trans*-retinal, all-*trans*-retinoic acid and 13-*cis*-retinoic acid more effectively stimulate STRA6-mediated retinol release and inhibit STRA6-mediated retinol loading than 9-*cis*-retinol, 9-*cis*-retinal and 9-*cis*-retinoic acid. **c** Schematic diagram of STRA6-mediated retinoid exchange using fenretinide as an example. **d** Schematic diagram of the effects of retinol-siRNA on STRA6 activity. When added extracellularly, retinol-siRNA has no effect on STRA6-mediated vitamin A uptake from holo-RBP due to its membrane-impermeable nature. However, retinol-siRNA potently stimulates STRA6-mediated retinol release and suppresses STRA6-mediated retinol loading when added to membranes that express STRA6

specificity in the interaction of retinoids with this vitamin A uptake machinery. The likely explanation is that the geometric shapes of the retinoids, which result from the configuration of their double bonds, affect their interaction with STRA6.

Fourth, from the perspective of the retinoids, STRA6 highly enhances the ability of these certain retinoids to release retinol from holo-RBP (Fig. 7). How does STRA6 enable these retinoids to stimulate retinol release from holo-RBP? The general model is that STRA6's interaction with RBP likely opens up the vitamin A exit end of RBP to allow retinol to come in or out. This interaction can explain the ability of STRA6 to stimulate both retinol release from holo-RBP and retinol loading into apo-RBP. Without STRA6, retinol does not come out of RBP fast enough to allow exchange with another retinoid. STRA6's specific interaction with RBP promotes retinol release from RBP and the exchange of other retinoids with retinol, if this retinoid can pass through STRA6. We have specifically demonstrated STRA6-catalyzed retinoid exchange with retinol in holo-RBP using radioactive experiments and mass spectrometry-based experiments (Fig. 6).

Fifth, experiments in this study support the interaction of retinoids with STRA6 facing the cytoplasmic side (Fig. 9). The long soluble and membrane-impermeable siRNA tail of the retinol-siRNA conjugate does not interfere with its ability to act on RBP/STRA6 when STRA6 is in the membrane form (Fig. 5) but does prevent its ability to affect STRA6-mediated vitamin A uptake in the live cell assay (Fig. 7). The fact that membrane-impermeable retinol-siRNA can only affect RBP/STRA6 from the cytoplasmic side is consistent with the interaction of retinoids with the RBP/STRA6 complex through a pore facing into the cell (Fig. 9d).

Sixth, experiments here also indicated that retinol is oriented with the β-ionone ring end first when it is loaded

into RBP by STRA6 (Fig. 9d). Like other all-*trans*-retinoids, fenretinide and retinol-siRNA stimulate STRA6's retinol release and inhibit STRA6's retinol loading (Figs. 1, 2 and 5). Compared to retinol itself, both fenretinide and retinol-siRNA have an intact β-ionone ring but a modified alcohol end. The fact that the very long conjugation at the alcohol end of retinol-siRNA does not prevent its effect on STRA6 is a clear demonstration of this orientation in retinoid movement through STRA6. This experiment is analogous to attaching RBP to Sepharose beads to demonstrate the RBP receptor's endocytosis-independent vitamin A uptake (Rask and Peterson 1976).

The finding that the cellular vitamin A uptake mechanism is a direct target of retinoids also has potential implications in retinoid-based therapy. It was discovered recently that vitamin A and all-*trans*-retinoic acid synergistically increase retinol uptake and retinyl ester storage in neonatal rat lung (James et al. 2010; Ross and Ambalavanan 2007; Ross and Li 2007; Wu and Ross 2010) and antibody production by the spleen (Tan et al. 2012). In addition to the ability of retinoic acid to stimulate STRA6 expression, the ability of exogenous retinoic acid to stimulate STRA6's activity may contribute to enhanced retinoid uptake.

It has been known for a long time that fenretinide has anticancer activity and much fewer side effects than retinoic acids (Ulukaya and Wood 1999), but its mechanism of action is not fully understood. Interestingly, certain cancer cells have more than 100-fold higher STRA6 expression levels (Szeto et al. 2001). STRA6 was originally known as a cancer cell surface marker before it was known as the RBP receptor. Because fenretinide can almost completely suppress STRA6-catalyzed retinol loading and strongly stimulate STRA6-catalyzed retinol release, its interaction with excessive STRA6 on cancer cells may cause retinoid overloading and contribute to fenretinide's anticancer activity.

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