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Investigating the Role of Tethering in Exosomal Secretion

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Abstract

Exosomes are a type of membrane vesicles utilized in intercellular communication. Exosomes are first packaged into larger membrane vesicles called multivesicular bodies (MVBs). When exosomes are secreted, the membrane of the MVBs fuses with the cellular membrane, and then the exosomes are released. Experimental observations suggest that the movement of the exosomes is hindered during this process; they require much longer time to leave the fusion site than they would if they were diffusing freely. A prevalent hypothesis is that the cause of this hindrance is attachment of the exosomes to the cell surface. Recent work suggests that tetherin, a transmembrane protein, might be the cause of this attachment. However, the evidence remains inconclusive. We present two computational models describing properties of this system, as well as experimental data exploring the possible involvement of the protein tetherin. The simulations describe properties of a theoretical tether, regardless of its actual nature or identity and the experimental data cannot be simulated without an exosomal tether.

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Abstract

Exosomes are a type of membrane vesicles utilized in intercellular communication. Exosomes are first packaged into larger membrane vesicles called multivesicular bodies (MVBs). When exosomes are secreted, the membrane of the MVBs fuses with the cellular membrane, and then the exosomes are released. Experimental observations suggest that the movement of the exosomes is hindered during this process; they require much longer time to leave the fusion site than they would if they were diffusing freely. A prevalent hypothesis is that the cause of this hindrance is attachment of the exosomes to the cell surface. Recent work suggests that tetherin, a transmembrane protein, might be the cause of this attachment. However, the evidence remains inconclusive. We present two computational models describing properties of this system, as well as experimental data exploring the possible involvement of the protein tetherin. The simulations describe properties of a theoretical tether, regardless of its actual nature or identity and the experimental data cannot be simulated without an exosomal tether.

Introduction

The interest in exosomes has grown dramatically in recent years as new research suggests they play a role in propagation of diseases. In particular, exosomes are involved in cancer propagation (Tkach & Théry 2016, Luga et al. 2012) as well as spreading of Alzheimer's disease (Rajendran et al. 2006). The role of exosomes in these various diseases is based on a similar mechanism- they shuttle cargo between cells, which impacts other cells in a way that causes the disease to spread or otherwise benefit.

The first step on the journey that the exosomes have to undertake to deliver their cargo is their secretion. Exosomes are produced inside of endosomes via invagination of their membrane. When many exosomes are produced, the endosome becomes a multivesicular body (MVB), which is also referred to as multivesicular endosome (MVE). The MVBs then travel towards the cellular membrane, onto which they dock. After docking, fusion of the cellular membrane and the MVB membrane occurs, leading to the release of the exosomes. Experimental observations suggest that the exosomes linger at the fusion site for several seconds after their release (Verweij et al. 2016, Mahmood et al. 2023). A common hypothesis explaining this is that the exosomes are attached to the cellular membrane. This hypothesis is supported by EM observations of attached exosomes (Edgar et al. 2016).

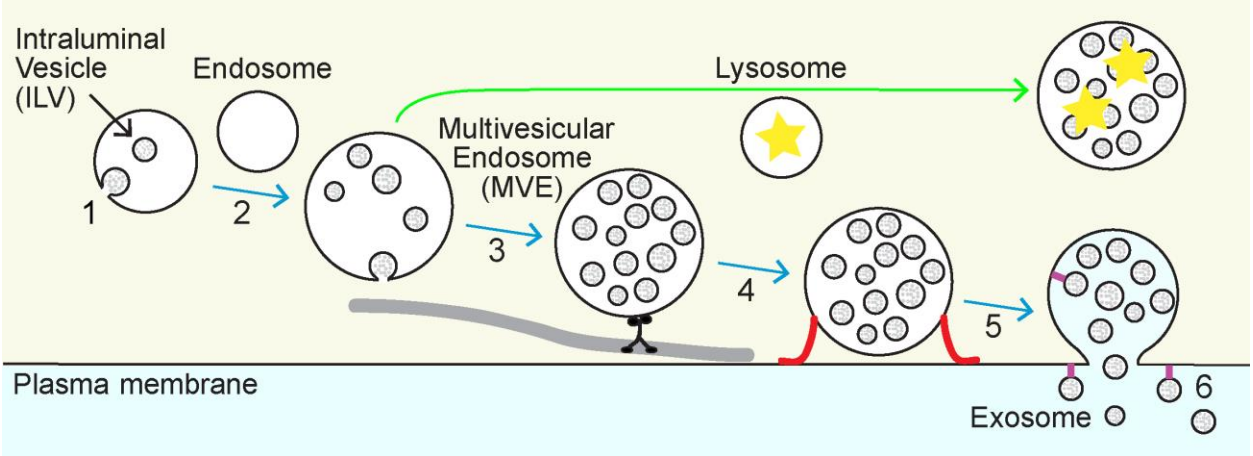

Figure 1: Exosome formation and secretion
1) Intraluminal vesicles are formed by invagination from endosomal membrane. 2) As more ILVs form, the endosome becomes a multivesicular endosome (MVE). 3) Multivesicular endosomes may either reform into lysosomes and be used for degradation of their content or be trafficked towards the cellular membrane to secrete the ILVs. 4) MVEs are docked onto the cellular membrane prior to membrane fusion and secretion. 5) ILVs are secreted as exosomes, some of which remain attached to the cellular membrane. (Adopted from Mahmood 2022)

Tetherin is a GPI-anchored membrane protein that has been mostly studied in virology, where it was shown to slow down the progress of infection of various viruses including HIV or SARS-CoV2 (Gupta et al. 2010, Stewart et al. 2021). Further research found that tetherin forms dimers with each tetherin molecule being inside of a different membrane. As a result, the membranes are tethered together. In the virological context, this manifests as either tethering of newly forming virions to the cellular membrane or tethering of virions to each other (Gupta et al. 2010, Neil et al. 2008, Van Damme et al. 2008). Whether tetherin tethers exosomes during their secretion in a similar manner is unclear. Some researchers have presented evidence suggesting that tetherin acts as an exosomal tether (Edgar et al. 2016), however other evidence suggests the opposite (Verweij et al. 2018). Tetherin is not the only molecule considered as a possible exosomal tether, other “candidates” include matrix adhesion proteins, fibronectins or integrins (Kalra et al. 2012, Kim et al. 2013, Keerthikumar et al. 2016). Overall, this area remains relatively poorly explored.

CD63 is membrane tetraspanin protein that is commonly used as an exosomal marker (Sung et al. 2020, Verweij et al. 2018). CD63-pHluorin is labelled by a pH-sensitive fluorescent probe. The probe does not fluoresce in acidic pH, but it is fluorescent in neutral pH. As a result, CD63-pHluorin can be utilized to visualize exosomal secretion. The inside of the MVBs is acidic, and consequently the probe is quenched while the exosomes are contained inside of them. When the exosomes are secreted, they are released into the buffer surrounding the cell, which has a roughly neutral pH. As a result, the probe starts to fluoresce, allowing for seeing when the secretion happens as well as tracking how the exosomes move shortly after the fusion. However, there is one important consideration; not all CD63 that can be tracked during exosomal secretion is found on exosomes. Between 30% and 34% is found on the membrane of the MVBs (Verweij et al. 2022). This portion of CD63 diffuses after the fusion as well; however, it moves at a slower rate than that found on exosomes, as it diffuses through the cellular membrane.

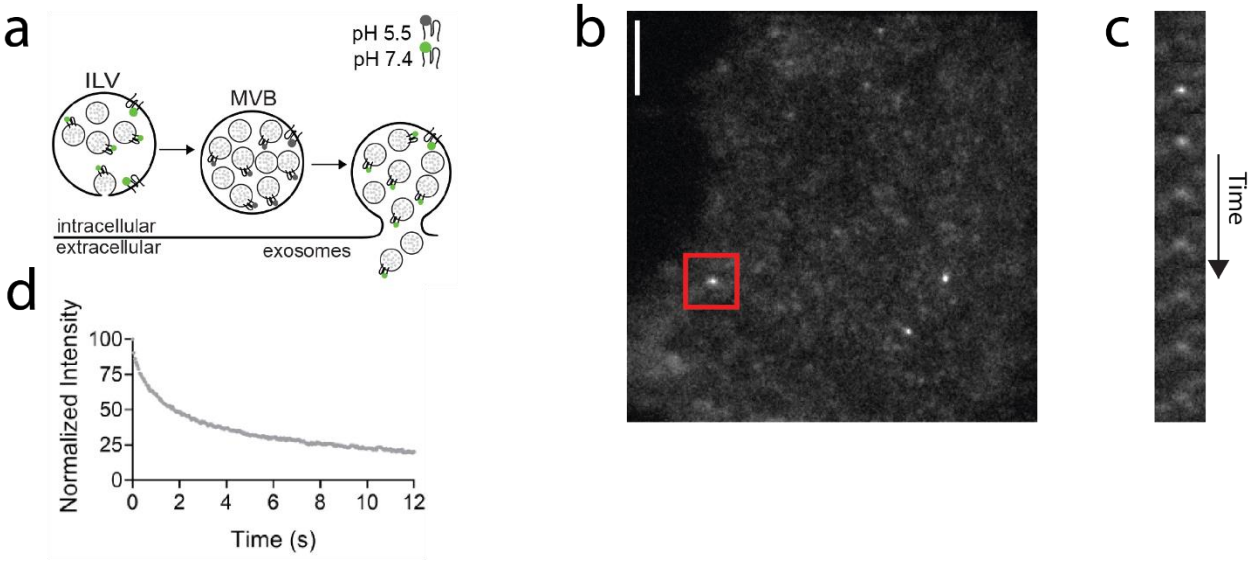

Figure 2: Experimental observations of MVB fusion and exosomal secretion
A) Exosomes are labelled with CD63-pHluorin, which fluoresces at neutral pH. The probe is quenched in acidic conditions. B) The release of exosomes manifests as a rapid increase in fluorescence (red box). C) Time development of the fluorescence in the area of the box (500ms between frames). D) Quantification of the average time development of the fluorescence intensity at the fusion site at 37°C (N= 97). Scale bar = 5µm

This work addresses two questions regarding exosomal secretion and their attachment to the cell surface. First, simulations based on experimental kinetic data of exosomal release were developed in MATLAB. These simulations describe the process independently of the character of the protein causing the attachment and suggest two possible models of exosomal release. Apart from the simulations, experimental work studying the effects of overexpression of tetherin in A549 cells was performed.

Results

To determine whether exosome attachment is a necessary part of exosomal secretion, and to gain insight into its mechanism, two random walk simulations were created using MATLAB, resulting in two different models of exosomal secretion. The simulations were compared to data obtained by TIRF imaging of CD63-pHluorin labelled A549 cells to assess their accuracy. In both models, there are three ways the particles can move from the fusion site. Some fraction of the particles represents untethered exosomes and diffuses at a rate corresponding to free diffusion of exosome-sized particles in water. The second part of the particles represents CD63-pHluorin that is in the endosomal membrane and diffuses at a rate corresponding to CD63 diffusing through cellular membrane (the diffusion coefficient was experimentally determined by FRAP). The final part of the particles represents tethered exosomes, that move differently in each of the models. The fractions of particles corresponding to each of the modes of movement were varied until a match with experimental data was reached. The particles move in steps, the direction of the steps is random, and its length is calculated based on the respective diffusion coefficient.

“Breaking tethers” model

In this model, the tethers are assumed to break with some half-life t_{half}. After the tethers break, the particles move with the same diffusion coefficient as free exosomes. The probability of a tether breaking increases exponentially in time. Figure 3 A shows a visualization of the possible movement types of the particles. Average experimental decays measured at 23, 27, 32 and 37°C were matched with the simulation by varying the fractions of particles diffusing from the fusion site as free exosomes, tethered exosomes or endosomal CD63, and the half-life of the tethers (Figure 3 B and C). The t_{half} values were 40s for 23°C, 10s for 27°C, 8.5s for 32°C, and 8s for 37°C. Additionally, a randomly picked set of the individual decays observed at 37°C was matched. The percentages of the different types of particle movement at 37°C were 40% tethered exosomes, 24% free exosomes, and 36% endosomal CD63 (Figure 3 D). An overview of the parameters used in the simulation can be found in Table 1.

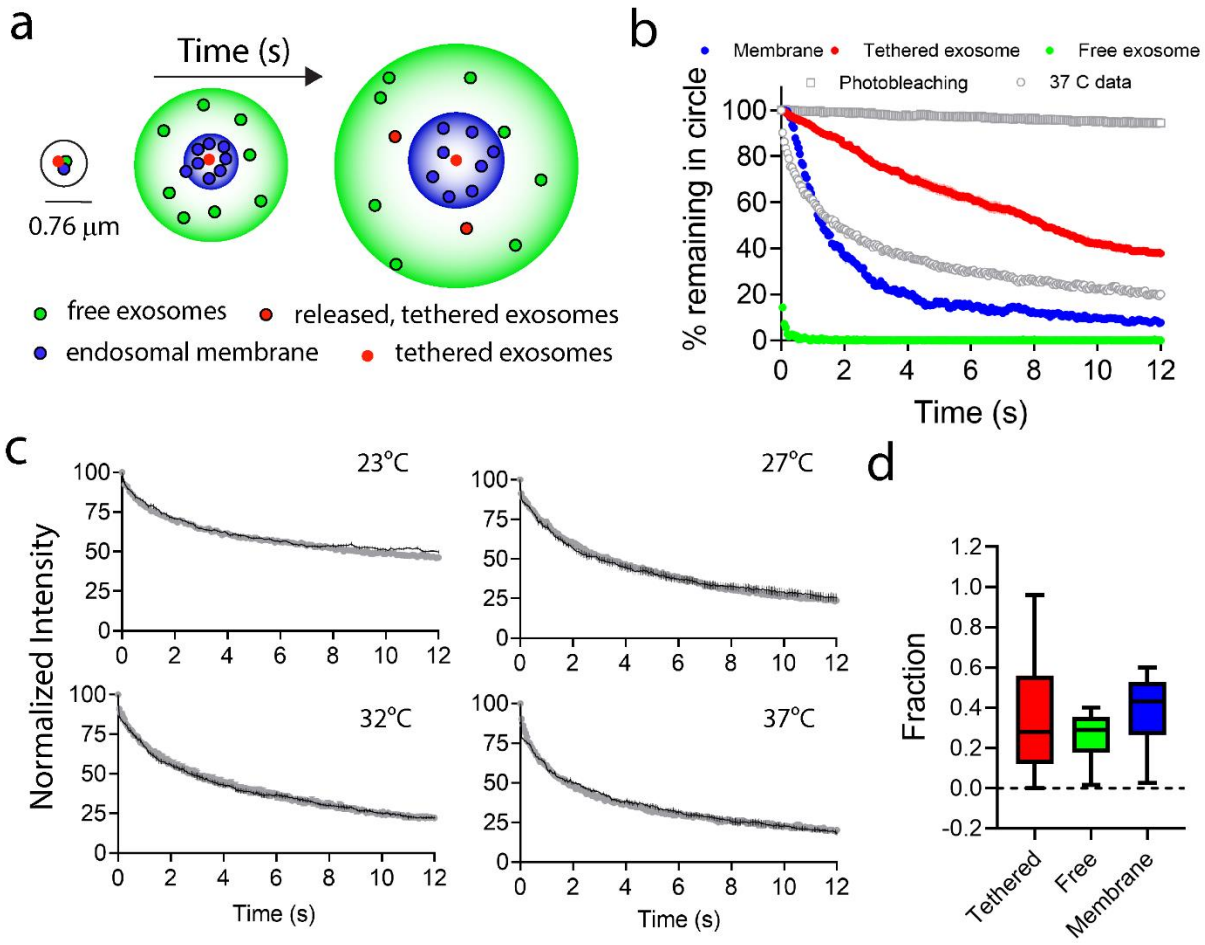

Figure 3: Exosome release can be modelled using breaking tethers
In the breaking tethers model, the tethers break with a set half-life, and the tethered exosomes are immobile until the tether breaks. A) Schematic of exosomal secretion, describing the three modes of fluorescence loss at the fusion site modelled in the simulation (tethered exosomes, free exosomes, and endosomal CD63). B) Simulated decays corresponding to the extremes of the three modes of fluorescence loss (100% tethered particles, 100% free particles or 100% endosomal CD63), the plot includes the average experimental 37°C decay and photobleaching. Both endosomal CD63 and free exosome decays are too fast to replicate the experimental decay. C) Simulated decays matching average experimental decays at 23, 27, 32, and 37°C. The matches were obtained by varying the fractions of the modes of fluorescence loss and the half-life of the tethers. D) Distribution of the fractions of particles corresponding to the best simulated matches to the individual experimental decays observed at 37°C (N= 20). (Edited and adopted from Mahmood et al. 2023)

“Mobile tethers” model

In the second model, the exosomal tethers are assumed to be permanent and mobile, diffusing with a diffusion coefficient that is selected as one of the inputs if the simulation. Similarly to the breaking tethers model, average experimental decays at 23, 27, 32 and 37°C were accurately matched by varying the fractions of tethered particles, free particles and particles representing endosomal CD63 (Figure 4 C). A randomly picked set of the individual decays at 37°C was also modelled, the average fraction of tethered exosomes, free exosomes, and endosomal CD63 were 40%, 24%, and 36% respectively. The theoretical diffusion coefficient of a tether was determined as 0.0015um^2/s, 0.003um^2/s, 0.004um^2/s, and 0.004um^2/s at 23, 27, 32 and 37°C respectively. The parameters used by both simulations for the best matches with the average experimental decays are summarized in Table 1.

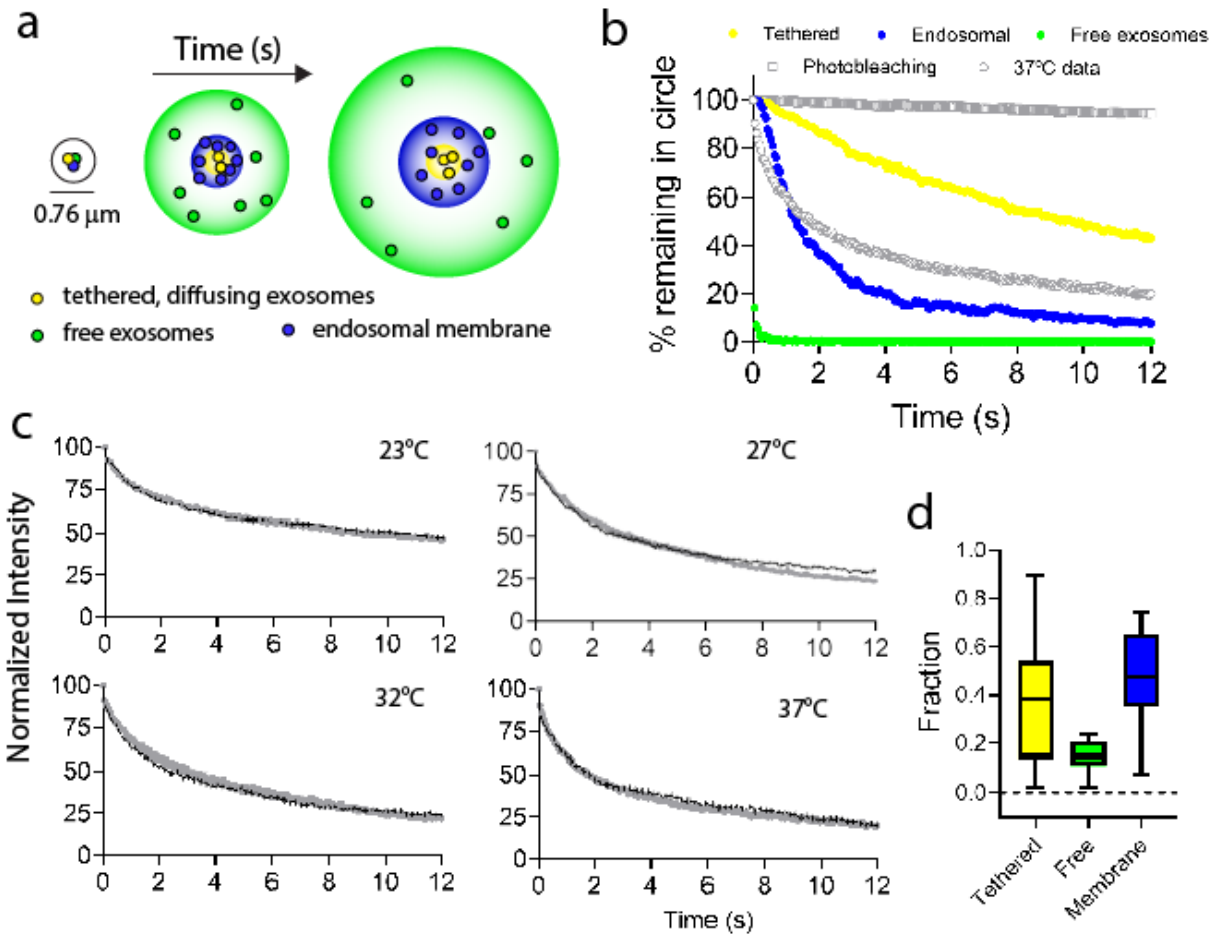

Figure 4: Replication of experimental decay data using the "mobile tethers" model
A) Schematic of exosomal secretion, describing the three modes of fluorescence loss at the fusion site modelled in the simulation (tethered exosomes, free exosomes, and endosomal CD63). In this model, the tethers are permanent, but mobile. B) Simulated decays corresponding to the extremes of the three modes of fluorescence loss (100% tethered particles, 100% free particles or 100% endosomal CD63), the plot includes the average experimental 37°C decay and photobleaching. C) Simulated decays matching average experimental decays at 23, 27, 32, and 37°C. The matches were obtained by varying the fractions of the modes of fluorescence loss and the diffusion coefficient of the mobile tethers. D) Distribution of the fractions of particles corresponding to the best simulated matches to the individual experimental decays observed at 37°C (N= 20). (Edited and adopted from Mahmood et al. 2023)

Table 1: Summary of simulation parameters for both models
The table includes all simulation parameters used in best matches between the models and experimental data. N events refers to the number of experimental decay traces used to make the average decay at the respective temperature. The diffusion coefficient of CD63 was determined by FRAP, the diffusion coefficient of free exosomes was calculated from the Stokes-Einstein equation as the diffusion coefficient of a freely diffusing exosome-sized particle. (Edited and adopted from Mahmood et al. 2023)

	D ($\mu m^2/s$)	**23°C**	**27°C**	**32°C**	**37°C**
N events	-	86	83	77	98
"Mobile tethers" model					
D_{tether}	varies	0.0015	0.003	0.004	0.004
Fraction tethered exosomes	varies	0.5	0.5	0.45	0.4
Fraction free exosomes	6.5	0.18	0.18	0.18	0.24
Fraction endosomal CD63	0.039	0.32	0.32	0.37	0.36
"Breaking tethers" model					
t_{half}	-	40	10	8.5	8
Fraction tethered exosomes	0	0.6	0.5	0.5	0.4
Fraction free exosomes	6.5	0.08	0.15	0.15	0.24
Fraction endosomal CD63	0.039	0.32	0.35	0.35	0.36

Tetherin-GFP mobility

Dynamic parameters of tetherin-GFP in the cellular membrane were determined using FRAP in order to assess its possible involvement in exosomal attachment. The average diffusion coefficient was determined to be 0.018um^2/s (Fig. 5 C) The data obtained in the experiment were then used as inputs in "mobile tethers" model and compared to the average experimental decay. This required a minor change in the original "mobile tethers" model to take into account the % of immobile particles (Figure 6 A). Comparing the simulated and the experimental decay after this modification resulted in a near-perfect match (Figure 6 B).

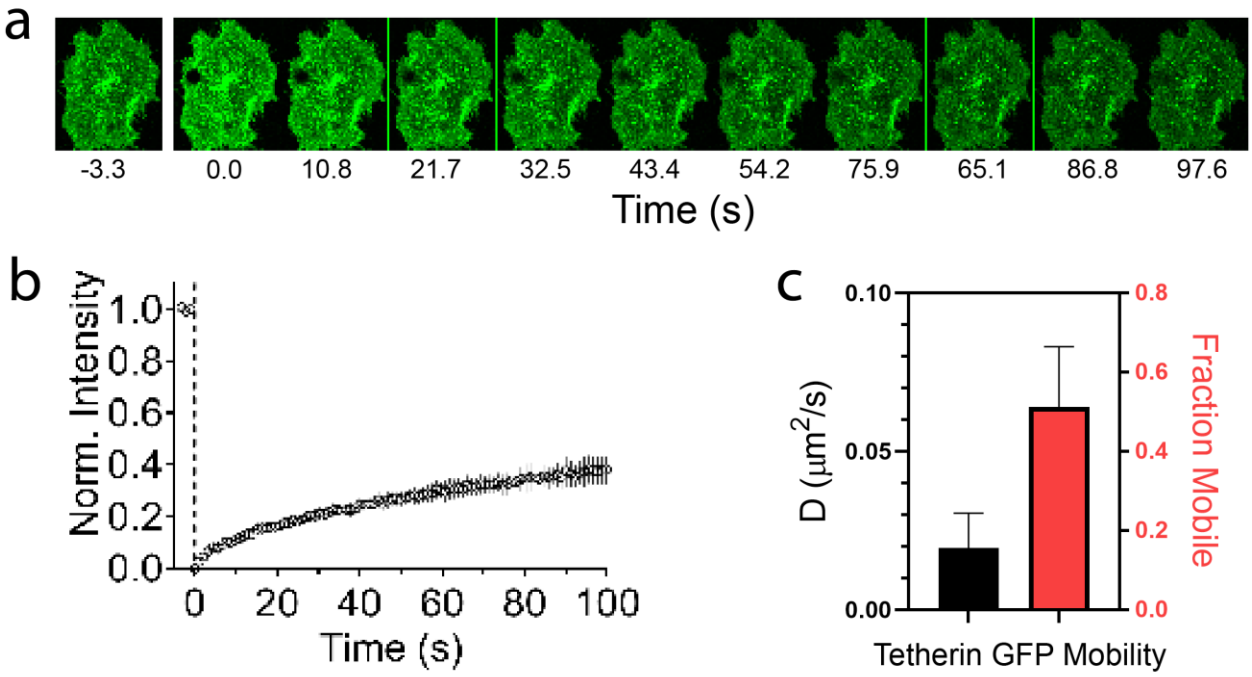

Figure 5: Tetherin-GFP mobility
A549 cells were transfected with tetherin-GFP and FRAP was performed (N= 10). A) Montage of the fluorescence recovery in cells during the experiment. B) A plot of fluorescence recovery in time. C) Diffusion coefficient and fraction mobile determined based on the experiment. The average diffusion coefficient was determined as 0.018um^2/s and the average fraction of mobile tetherin as 51%.

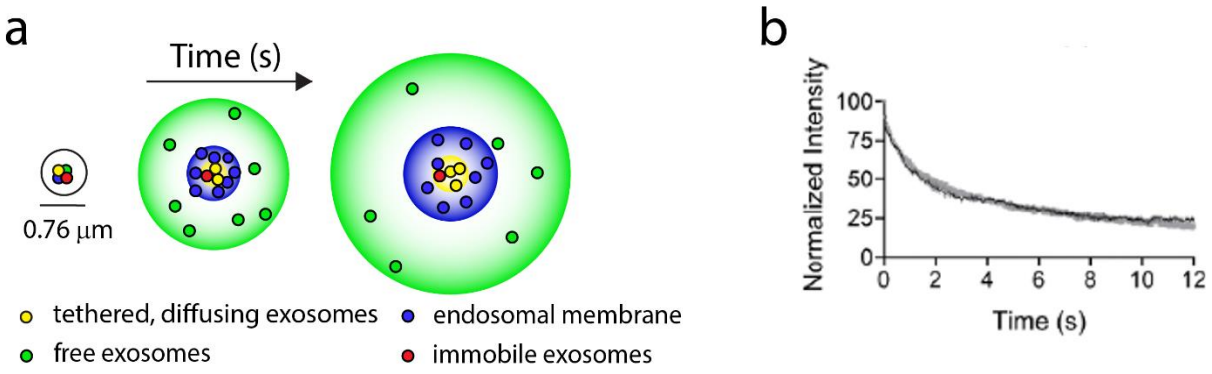

Figure 6: Experimental fluorescence decays can be replicated using modified "mobile tethers" model and FRAP results
The mobile tethers model was modified to include a fraction of completely immobile particles. A) A cartoon describing the modes of fluorescence loss in the model. B) A match between experimental fluorescence decays and the simulation was obtained by using the diffusion coefficient and fraction of immobile particles determined by FRAP of tetherin-GFP (D= 0.018um^2/s, fraction mobile= 51%).

Tetherin distribution in A549 cells

To describe the distribution of tetherin in A549 cells, permeabilized, fixed cells transfected with HA-tetherin and CD63-GFP were imaged using confocal microscopy. Tetherin was labelled using HA-Tag antibody. Tetherin was heavily overexpressed (the amount of used DNA was 4x higher than in all other experiments). The resulting images clearly show that tetherin localizes to the cellular membrane.

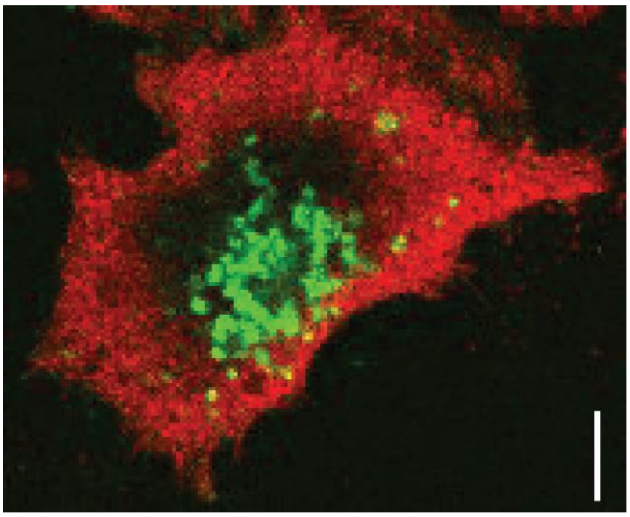

Figure 7: Tetherin is localized to the cellular membrane
A549 cells were transfected with tetherin HA and CD63 GFP, fixed, permeabilized, and treated with red HA-Tag antibody. The cells were then imaged with confocal microscopy. The images show average intensity over z-stacks. Scale bar= 5µm

Tetherin and CD63 colocalization

For tetherin to act as an exosomal tether, it is necessary that it is found on exosomes as well as on the MVB membrane. To determine whether that is the case, colocalization of exosomes (labelled by CD63) and tetherin was analyzed in A559 cells transfected with tetherin-HA, CD63-mCherry. HA intrabody was used to label tetherin-HA.

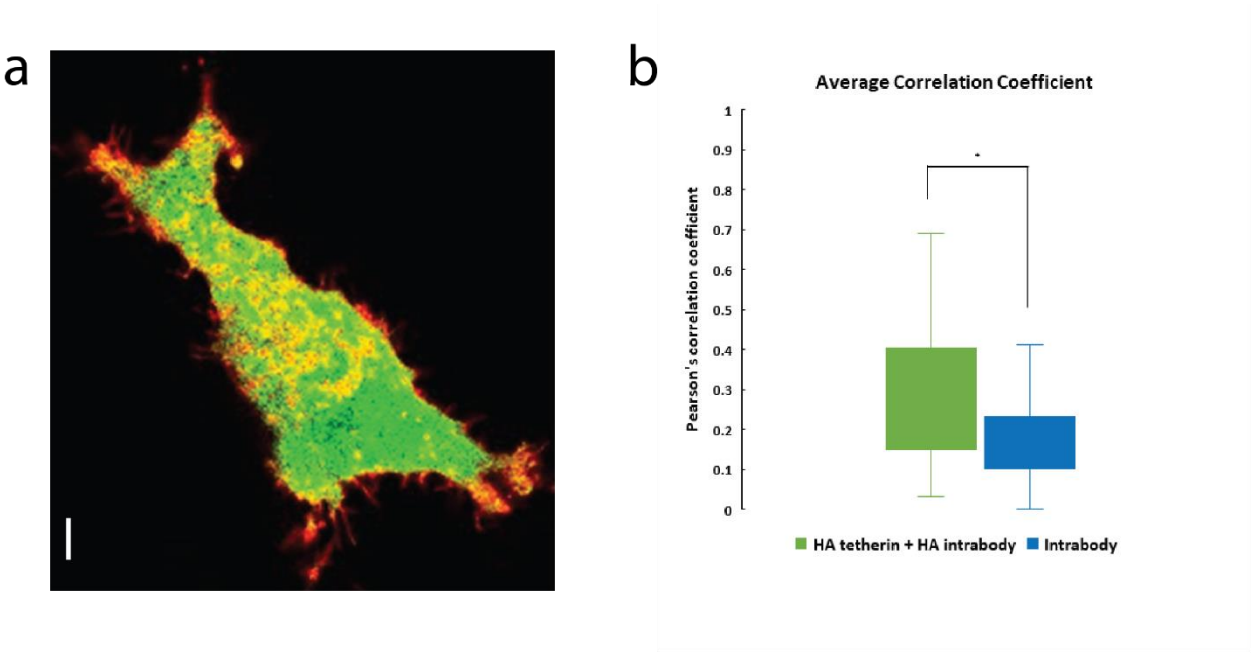

Figure 8: Tetherin-HA colocalized with CD63-mCherry
A) A549 cells were transfected with tetherin-HA, CD63-mCherry and HA intrabody. B) The HA intrabody is significantly more correlated with CD63 in the samples transfected with tetherin-HA than in the negative control (cells transfected with CD63-mChery without tetherin-HA), suggesting that tetherin-HA and CD63 are colocalized (p= 0.029). Scale bar= 5µm

Tetherin Western Blot

To confirm whether overexpression was achieved, western blot was used. A549 cells transfected with tetherin-GFP were used in this experiment. The experiment confirmed that the transfection results in overexpression of tetherin (Figure 8). Additionally, the transfection causes a small amount of dimeric tetherin to be visible on the blot. The results are consistent with published blots of tetherin (Hammonds et al. 2012).

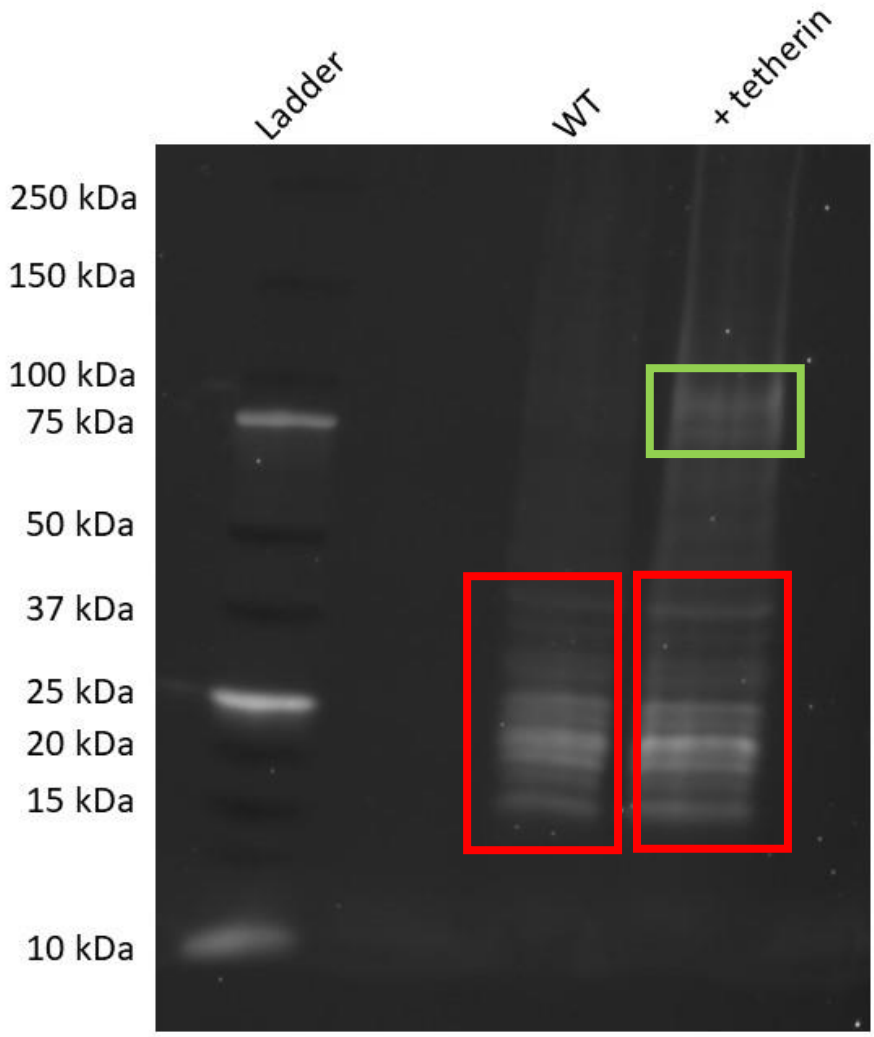

Figure 9: Verifying tetherin overexpression with western blot
A549 cells transfected with tetherin-GFP were lysed and western blot was performed to assess the difference between tetherin-overexpressed cells and wild type cells. Tetherin monomers can be seen in red boxes, tetherin dimers in green. Tetherin dimers are visible in the transfected cells (+ tetherin), but not in the wild type samples. The increase in monomer intensity confirms the effects of the transfection. Many bands can be seen due to glycosylation of the protein.

Discussion and future directions

Regardless of the nature of the tethers, the results of the simulations yield two models of exosome attachment. The results of the breaking tethers model provide several interesting insights. Probably most importantly, the simulation was able to accurately replicate the experimentally observed decays. The value of the tether half-life and its exact meaning are of interest. The most intuitive way to think about the meaning of the t_{half} would be the most literal one- a tether breaking in time. However, considering how the simulation works, the number may relate to other things as well. In the simulation, the particles representing the tethered exosomes remain static until their tether "breaks", the probability of this breaking is determined based on the half-life. This approach could consider other factors contributing to the slower movement than cell attachment. For example, it is likely that the diffusion of exosomes is hindered by collisions with the cellular membrane as well as collisions among exosomes themselves after their release.

Similarly to the "breaking tethers" model, it was possible to replicate the experimental data at the four temperature levels using the "mobile tethers" model. The fractions of the free exosomes, tethered exosomes, and endosomal CD63 are comparable between the two models. The percentage of endosomal CD63 predicted by both simulations is close to the values of 30-34% reported in literature (Verweij et al. 2022), which increases the credibility of the other results.

The parameter relating to the speed of the decay (t_{half} or D_{tether}) shows a dramatic dependence on temperature in both models. This dependence seems intuitive in the case of diffusion coefficient; however, its meaning is less clear in the case of the tether half-life. Also, the fractions of the modes of fluorescence decay vary with the temperature, though less dramatically. This

complicates the hypothesis that tetherin acts as the exosomal tether. Tetherin dimers are GPI-anchored and held together with disulfide bonds. Formation or destruction of tetherin tethers, or a physical removal of a tetherin dimer from a membrane due to a relatively small change in temperature thus seem unlikely. These differences might be easier to explain with some other molecule, depending on the particular attachment mechanism.

The fact that both of the models can reliably replicate the experimental data raises a question- which one is correct? In the "breaking tethers" model the tethers are assumed to be permanent (or at least long-term), which would imply that the total number of exosomes secreted in time would decrease with increasing tethered fraction. Consequently, quantifying the amount of released exosomes in cells expressing increased or decreased amounts of a suspected tethering molecule, and comparing those to wild type cells, could provide answers.

The results of the FRAP experiment and the ability to produce a good simulated decay with the modified "mobile tethers" model provide some evidence pointing towards the "mobile tethers" model being correct with tetherin acting the tether. With this in mind, preliminary experiments dealing with exosome quantification in cells overexpressing tetherin are ongoing. While the results are not clear yet, they could potentially answer both questions- which of the models is valid as well as whether tetherin truly acts as an exosomal tether.

Additional experiments show that tetherin and exosomes are colocalized, that tetherin and exosomes are in the same places at the same time. While this does not prove that tetherin tethers the exosomes, it is consistent with the hypothesis.

The confirmation of tetherin overexpression via western blot does not bring a lot of meaningful conclusions on its own; however, the results of these experiments can be used in future work

dealing with exosome quantification to evaluate the level of overexpression achieved by the transfection.

Methods

MVB Fusion events modelling

MVB fusion events were modelled as a point source of 100 CD63-pHluorin molecules deposited randomly into a circle with a diameter of 0.5µm. After the deposition, the molecules start moving and can escape a circle with a diameter of 0.76µm, which corresponds to the size of an experimental fusion site. The molecule can move from the deposition site in three different ways: 1) Free exosomes, that move at a rate of 6.5$\mu m^2/s$. This diffusion coefficient was determined using the Stokes-Einstein equation $D= k_bT/(6\pi\eta r)$, where k_b is the Boltzmann constant, T is 310K, η is the aq. Buffer viscosity (o.69cP), and r is the radius of an exosome, which ranges between 15 and 60nm. For the purposes of the modelling, 50nm was used. 2) Tethered exosomes, which are treated in two different ways in the models. In the "mobile tethers" model, the tethers diffuse with a diffusion coefficient varied between 0 and 0.0018um^2/s. In the "breaking tethers", the tethered exosomes are immobile, until a tether breaks. The time at which the tether breaks is determined by its t_{half}, which was varied between 0 and 50s. An exosome is allowed to have only one tether at a time. 3) In both models, there is also a fraction of CD63 molecules present on the endosomal membrane, this fraction was constrained to approximately 30-40%. The simulation uses 50µs steps during which the molecules move as described above, and the location of each molecule is recorded every 50ms (100ms was used for 23°C data to obtain longer data sets). The simulations were run for 12.5 to 25s, and the number of molecules remaining in the 0.76µm circle was recorded. The fractions of tethered exosomes, free exosomes, endosomal CD63, and

the t_{half} or D_{tether} were varied in both models in order to find the best matching parameters for the average experimental decays. For the individual decays, 20 experimental decays measured at 37°C were randomly picked. The ratio of endosomal CD63 to free exosomes was determined by matching the first 10 frames of the model to the experimental data (the tethered component decays too slowly to contribute to the decay at this stage). t_{half} and D_{tether} of the best matches of average 37°C experimental were used. The tethered fraction was varied in increments of 2% from 0 to 100%, the remaining molecules were split between endosomal CD63 and free exosomes based on the previously determined ratio. The absolute value of differences between the simulated and experimental decay was used to find the best match in all simulations.

Cell Culture

A549 cells were cultured in T75 flasks (CELLTREAT, Life Science Products, Frederick, CO) in DMEM modified with 10% fetal bovine serum. The cells were cultured in a humidified 37°C, 5% CO_2 incubator. For imaging, the cells were transferred to LabTek 8-well dishes.

Cell transfection

200ul of DMEM media containing cells released using trypsin was loaded into a LabTek 8-well plate. A transfection mixture containing 0.6µl of L3000 reagent (Thermo Fisher Scientific, Waltham, MA), 1.2µl of P3000 (Thermo Fisher Scientific, Waltham, MA), and 0.7µl of 100ng/µl DNA solution was prepared. 2.5µl of the mixture was used per well.

Cell fixing and permeabilization

Cells were fixed by applying 10% paraformaldehyde solution for 10 minutes. Permeabilization was performed using 0.5% Triton X-100 solution for another 10 minutes. Cells were washed 3 times with PBS before imaging.

Confocal microscopy and FRAP

Confocal imaging and FRAP were performed using point-scanning confocal microscopy (Olympus Fluoview 3000). Averages of z-layers are used in this work. For FRAP of tetherin-GFP, a 488nm laser was used to bleach a circle with a diameter of 16 pixels (3.98μm). The fluorescence recovery was then used to determine tetherin-GFP's diffusion coefficient and fraction of mobile particles. Detailed description of the analysis of the FRAP data can be found in past published work of the Knowles Lab (Black et al. 2014). Santa Cruz Biotechnology HA-Tag Antibody (F-7): sc-7392 was used in the images of tetherin distribution.

Correlation coefficient determination

Pearson's correlation coefficient was calculated for each z-layer in the cell using a MATLAB code written by the Knowles Lab. The correlation coefficient of the individual layers was then averaged. An independent t-test was used to test for significance.

Western blot

Cell lysate was obtained from A549 cells grown to approximately 80% confluence. The cells were released using trypsin and spun down in a test tube at low speed for 5 minutes. The cell pellet was then washed by resuspending in 3ml of cold PBS and spinning for 5 minutes, the wash was repeated 3 times. The cell pellet was then left to incubate with 200ul of lysis buffer (50mM Tris base, 150mM NaCl, 1% v/v Triton X-100, pH=7.8) for 30 minutes in an ice bucket. The cell lysate was then mixed with an equal amount of 2x Laemmli Sample Buffer (Bio-Rad), homogenized using a syringe and centrifuged at 14000g for 15 minutes. The supernatant was then loaded into a precast 4-15% stain-free gel (Bio-Rad, cat. #4568083). The samples were then run at 90V for 2 hours. The samples were transferred to a nitrocellulose membrane (Bio-Rad, cat.

#1620215) at 250V for 4 hours. The membrane was blocked with 5% BSA solution for 2 hours and incubated with a 1:400 solution of the primary antibody overnight. For tetherin, BST-2 (E-4) mouse monoclonal antibody was used (Santa Cruz Biotechnologies, sc-390719). The primed membrane was then washed five times for 5 minutes with a TBST buffer (20mM Tris base, 150mM NaCl, 0.1% Tween 20, pH=7.5). Finally, the primed membrane was incubated for 2 hours in a 1:400 secondary anti-mouse antibody solution (Santa Cruz Biotechnologies, sc-516178). The membrane was then washed two times with TBST buffer and one more time with TBS buffer (20mM Tris base, 150mM NaCl, pH=7.5). A Bio-Rad ChemiDoc MP Imaging System was used for imaging.

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Contributions

Dr. Anarkali Mahmood performed the experimental data collection used in the development of both models.

Dr. Alan Weisgerber contributed to the refinement of western blot technique and FRAP experiments.

Dr. Michelle Knowles from the Department of Chemistry and Biochemistry was the advisor for this project.

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