Published in final edited form as:

Methods Mol Biol. 2020 ; 2109: 185–197. doi:10.1007/7651_2019_239.

Biotin Identification Proteomics in Three-Dimensional Organotypic Human Skin Cultures

Calvin J. Cable,

Department of Dermatology, Northwestern University, Chicago, IL, USA

Nihal Kaplan,

Department of Dermatology, Northwestern University, Chicago, IL, USA

Spiro Getsios,

Department of Dermatology, Northwestern University, Chicago, IL, USA

Paul M. Thomas,

Department of Molecular Biosciences, Northwestern University, Chicago, IL, USA; Northwestern Proteomics, Northwestern University, Chicago, IL, USA

Bethany E. Perez White

Department of Dermatology, Northwestern University, Chicago, IL, USA, Skin Disease Research Center, Northwestern University, Chicago, IL, USA

Abstract

Biotin identification (BioID) proteomics facilitates the unbiased detection of protein interaction neighborhoods in live cells. The BioID technique relies on the covalent biotin alteration of vicinal proteins by a modified bacterial biotin ligase. The biotin ligase is fused to a protein of interest to identify putative protein-protein interactions. Here, we describe the adaptation of this technique for use in three-dimensional epidermal cultures. Due to the covalent biotin modification of proteins, our protocol allows for the complete solubilization of the total cellular protein content in differentiated keratinocytes. Thus, a comprehensive network of potential interactors of a protein of interest can be mapped.

Keywords

3D skin culture; Keratinocytes; Epidermis; Protein-protein interaction; Biotin-identification proteomics; BioID

1 Introduction

The epidermis is a stratified squamous epithelium consisting of keratinocytes in well-defined states of proliferation, differentiation, and death. The balance of these cell processes requires the strict coordination of a variety of cellular signal transduction pathways to maintain tissue homeostasis [1]. However, our understanding of the signals that drive the complex intracellular and tissue-level architecture of epidermis remain largely unknown [2, 3]. While the use of proteomic discovery approaches has increased our understanding of the protein content of skin compartments [4-6], the application of proteomics to gain insight into the

signaling networks that maintain epidermal homeostasis is limited. Primary epidermal keratinocyte cell cultures, submerged two-dimensional (2D) and three-dimensional (3D) organotypic cultures, have proven to be a powerful tool in elucidating mechanisms driving epidermal morphogenesis and the balance between proliferation and differentiation. 3D skin culture models histologically and biochemically recapitulate all the layers of human skin and can be used for skin disease modeling [7, 8]. Like human epidermis, 3D cultures have an extensive cytoskeleton, mainly comprised of keratin intermediate filaments, which is insoluble in conventional extraction buffers. Further, as keratinocytes become terminally differentiated, there is increased protein cross-linking to form the epidermal barrier system. These characteristics of the epidermis necessitate alternative proteomic approaches to fully catalog relevant protein interaction networks that dictate epidermal cell fate.

Biotin identification (BioID) proteomics is a technological advance, first described by Roux and colleagues, which allows for the real-time detection of protein interaction networks in live cells [9,10]. In this approach, a protein of interest is fused to a bacterial biotin ligase that has been modified to promiscuously and covalently biotinylate accessible lysine residues of vicinal proteins in the presence of excess biotin. Biotinylated proteins are efficiently removed from the cellular milieu with streptavidin precipitation. The key advantages of this technique are that (1) there is no temporal bias for protein-protein interactions and (2) weak or transient interactions are not affected by the choice of extraction buffer. Here, we describe a modified protocol uniquely suited to protein identification in 3D epidermal cultures and differentiated 2D submerged cultures [11]. This protocol involves the use of a urea sample buffer that efficiently solubilizes all the protein content of epidermal keratinocytes while maintaining the covalently bound protein-biotin complexes for streptavidin precipitation and preparation for protein identification by mass spectrometry of tryptic peptides. As such, our adapted BioID approach allows for the identification of tissue-level protein interaction networks in the highly cross-linked and keratinized intracellular environment of epidermis.

2 Materials

2.1 Biotin Stock Solution

Biotin stock solution, 50 mM (1000×): add 4 mL of 1 N NaOH (prepared in molecular grade water suitable for cell culture) directly to bottle of biotin powder (MW: 244.31; 100 mg bottle). Pipette up and down to dissolve biotin. Add 4.19 mL molecular grade water suitable for cell culture to bring the final volume to 8.19 mL. Sterile filter biotin stock solution with a 0.22 µm syringe filter, and store at 4 °C for up to 1 month (see **Note 1**).

2.2 Check Keratinocytes for Biotinylating Activity

1. Keratinocytes expressing BirA* fusion construct and control keratinocytes
2. Glass coverslips (18 mm diameter) in a 12-well plate
3. Optimum cutting temperature (OCT) compound
4. Cryostat for sectioning OCT-embedded tissue
5. Biotin stock solution

6. 1× phosphate-buffered saline (PBS)
7. 4% methanol-free paraformaldehyde (PFA): add 10 mL of 16% PFA, 4 mL 10× PBS, and 26 mL molecular grade water
8. 5% normal goat or donkey serum in 0.3% (v/v) Triton X-100 in 1× PBS
9. 1% normal goat or donkey serum in 1× PBS
10. Tag-specific antibody (hemagglutinin (HA)-tag or myc-tag, as appropriate)
11. Fluorophore-conjugated streptavidin
12. Antibody targeting BirA*-tagged protein (optional)
13. 1× 4',6-diamidino-2-phenylindole (DAPI)
14. Fluorophore-conjugated secondary antibodies
15. Mounting media suitable for fluorescence microscopy
16. Microscope slides
17. Cover glass (22 × 50 mm)

2.3 Urea Sample Buffer

1. Deionized urea, 10 M (MW: 60.06): (*this process can be scaled down proportionately*) Weigh out 300 g of urea (volume of this amount is 350–400 mL). Add distilled water at 100 mL increments to a final volume of 500 mL (urea + water). Dissolve urea by stirring on a magnetic stir plate with gentle heat (*see Note 2*). When dissolved, turn off heat, cool to room temperature, and add 50 g mixed bead resin-hydrogen and hydroxide form (10 g/100 mL 10 M urea). Continue stirring for up to 60 min until urea is sufficiently deionized (*see Note 3*). Crude filter the deionized urea with Whatman paper to remove the mixed bead resin. The deionized urea can be aliquoted and stored at 4 °C for no longer than 1 month or at –20 to –80 °C for up to 5 years.
2. 100% glycerol.
3. 20% (w/v) sodium dodecyl sulfate.
4. Tris buffer, 1.875 M, pH 6.8.
5. 1% (w/v) pyronin Y in water.
6. β-mercaptoethanol.
7. 27 gauge needle with a 3 mL syringe.

2.4 Amido Black Protein Assay

1. Naphthol blue black dye
2. 100% methanol
3. Acetic acid

4. Nitrocellulose membrane
5. 50% methanol in distilled water
6. Plate reader with 595 nm optical density filter
7. Bovine serum albumin
8. 0.1 N NaOH
9. 1.5 mL tubes
10. 96-well clear flat bottom microplate

2.5 Streptavidin Precipitation

1. TEXN buffer: 0.2 M Tris, pH 7.5, 5 mM EDTA, 1% Triton X-100, 150 mM NaCl
2. Streptavidin-conjugated magnetic beads (Thermo Fisher Pierce Streptavidin Magnetic Beads catalog #88817 or #88816; *see Note 4*)
3. Magnetic stand
4. Tube rotator
5. 2 mL tubes

2.6 Washing Beads After Precipitation

1. Wash buffer 1: 2% (w/v) sodium dodecyl sulfate
2. Wash buffer 2: 0.1% (w/v) deoxycholic acid (sodium deoxycholate; store deoxycholate stocks protected from light and discard if cloudy), 1% (v/v) Triton X-100, 1 mM EDTA, 500 mM NaCl, 50 mM HEPES, pH 7.5
3. Wash buffer 3: 0.5% (w/v) deoxycholic acid, 0.5% (v/v) NP-40, 1 mM EDTA, 250 mM LiCl, 10 mM Tris-Cl, pH 7.4
4. Tris-buffered saline with Tween (TBS-T): 50 mM Tris, pH 7.4, 150 mM NaCl, 0.1% (v/v) Tween-20
5. Tris-buffered saline (TBS): 50 mM Tris, pH 7.4, 150 mM NaCl
6. 50 mM Tris, pH 7.4

2.7 Isolation of Peptides for Mass Spectrometry

1. 100 mM ammonium bicarbonate (analytical or chromatography grade) prepared in HPLC grade water and sterile-filtered with a 0.22 μ m filter
2. 10 mM dithiothreitol prepared in 100 mM ammonium bicarbonate (*see Note 5*)
3. 100 mM iodoacetamide prepared in 100 mM ammonium bicarbonate (*see Note 6*)
4. Eppendorf Protein LoBind tubes, 1.5 mL

5. Sequencing grade modified trypsin, porcine (Promega, catalog #V5111)
6. Formic acid, ACS grade or higher
7. 1.5 mL O-ring gasket tubes

2.8 Check Samples for Mass Spectrometry

1. Elution buffer: 2% sodium dodecyl sulfate, 0.4 M deionized urea (as in Subheading 2.3)
2. Pink glycerol: 50% glycerol with 0.1% pyronin Y
3. Materials and equipment for western blot
4. TBS-T (as in Subheading 2.6)
5. 10% blotting grade fat-free milk prepared in TBS-T
6. Horseradish peroxidase-conjugated streptavidin antibody
7. Peroxide-based chemiluminescent reagent

3 Methods

Upstream steps should be completed before beginning this protocol, including molecular cloning of the BirA* enzyme to fuse to the protein of interest, molecular cloning into an appropriate expression vector, cell transduction of the BirA* fusion protein and appropriate control (see **Note 7**), cell culture, and any pre-treatments (calcium switch, drug, ligand, etc.).

3.1 Check Cells for Biotinylating Activity

Check biotin stock solution and the expression pattern and biotinylating activity of BirA* fusion constructs in 2D or 3D primary epidermal cultures (Fig. 1).

3.1.1 Biotinylating Activity in 2D Cultures

1. Plate keratinocytes onto coverslips in wells.
2. Treat cells with biotin (50 μ M final concentration) or with vehicle for 24 h.
3. Rinse cells briefly with PBS.
4. Fix cells in 4% PFA for 15 min.
5. Rinse cells 3 $\times$ 5 min with PBS.
6. Block with 5% normal goat or donkey serum for 45 min.
7. Incubate with tag-specific antibody (optional: add primary antibody targeting BirA*-tagged protein) for 1 h at room temperature in 1% normal goat or donkey serum in PBS as diluent.
8. Rinse in PBS 3 $\times$ 15 min.
9. Add appropriate fluorophore-conjugated secondary antibodies with fluorophore-conjugated streptavidin for 30 min at room temperature.

10. Rinse coverslips 3×15 min with PBS.
11. Counterstain with DAPI for 5 min.
12. Mount on a microscope slide.
13. Image using a fluorescent microscope.

3.1.2 Biotinylating Activity in 3D Cultures

1. Embed 3D cultures as frozen tissue samples in OCT compound.
2. Section tissue longitudinally at $4 \mu\text{m}$ with a cryostat and mount to a microscope slide.
3. Rinse cells briefly with PBS.
4. Fix cells in 4% PFA for 15 min.
5. Rinse cells 3×5 min with PBS.
6. Block with 5% normal goat or donkey serum for 45 min.
7. Incubate with tag-specific antibody (optional: add primary antibody targeting BirA*-tagged protein) for 1 h at room temperature in 1% normal goat or donkey serum as diluent.
8. Rinse in PBS 3×15 min.
9. Add appropriate fluorophore-conjugated secondary antibodies with fluorophore-conjugated streptavidin for 30 min at room temperature.
10. Rinse coverslips 3×15 min with PBS.
11. Counterstain with DAPI for 5 min.
12. Mount and coverslip.
13. Image using a fluorescent microscope.

3.2 Cell Treatment and Harvest for Mass Spectrometry

1. To treat cultures for downstream mass spectrometry analysis, add biotin stock solution to a final concentration of $50 \mu\text{M}$ in the culture media for 24 h in 2D culture and for 72 h in 3D culture.
2. Make the appropriate volume of urea sample buffer. For 10 mL, combine 8 mL 10 M deionized urea, 1 mL glycerol, $500 \mu\text{L}$ 20% (w/v) sodium dodecyl sulfate, $320 \mu\text{L}$ 1.875 M Tris (pH 6.8), and $100 \mu\text{L}$ 1% pyronin Y. Prepared USB can be stored at room temperature. Just before use, add 5% β -mercaptoethanol to the necessary volume of urea sample buffer.
3. Lyse and harvest cells in urea sample buffer such that the final protein concentration is $4\text{--}5 \mu\text{g}/\mu\text{L}$ (*see Note 8*).
4. Syringe cell lysate through a 27 gauge needle with 3 mL syringe (*see Note 9*).

5. Determine protein concentration by the amido black protein assay.

3.3 Amido Black Protein Assay (See Note 10)

1. Prepare amido black dye: 0.1% (w/v) naphthol blue black in a solution of 30% methanol and 10% acetic acid.
2. Prepare bovine serum albumin protein standards in urea sample buffer.
3. Spot 5 μ L of each sample onto nitrocellulose membrane, including bovine serum albumin protein standards.
4. Incubate protein-spotted nitrocellulose in 50% methanol/water solution for 5 min with gentle agitation.
5. Pour off methanol solution and incubate protein-spotted nitrocellulose in amido black solution for 5 min with gentle agitation.
6. Pour off amido black solution and rinse protein-spotted nitrocellulose in 50% methanol/water solution until background nitrocellulose is almost white again.
7. Cut spots out of nitrocellulose and place each spot in a 1.5 mL tube
8. Elute dye in 500 μ L 0.1 N NaOH for 10 min. Vortex briefly once.
9. Add 200 μ L eluate to a 96-well microplate.
10. Read the optical density at 595 nm.
11. Interpolate protein concentration from the bovine serum albumin protein standards.

3.4 Streptavidin Precipitation (See Note 11)

1. Prepare streptavidin magnetic beads: gently mix beads by inversion until completely resuspended. Take 100 μ L of beads per sample, plus 50–100 μ L extra. Using the magnetic stand, wash three times in TEXN buffer, mixing gently by inversion until resuspended each time. Finally, resuspend beads in TEXN buffer in the initial suspension volume.
2. Put 3 mg of sample lysate into a 2 mL.
3. Add TEXN buffer to a final volume of 1.8 mL (*see Note 8*).
4. Add 100 μ L of prepared streptavidin beads to each sample tube.
5. Precipitate biotinylated proteins overnight at 4 °C overnight on a tube rotator.

3.5 Washing Beads After Precipitation

1. Following precipitation, isolate beads, discard supernatant, and wash beads in 1 mL wash buffer 1 for 10 min at room temperature on a rotator.
2. Isolate beads, discard supernatant, and repeat another wash in 1 mL wash buffer 1 for 10 min at RT on a rotator.

3. Isolate beads, discard supernatant, and wash in 1 mL wash buffer 2 for 10 min at RT on a rotator.
4. Isolate beads, discard supernatant, and wash in 1 mL wash buffer 3 for 10 min at RT on a rotator.
5. Isolate beads, discard supernatant, and wash briefly by inversion with 1 mL TBS-T.
6. Isolate beads, discard supernatant, and wash briefly by inversion with 1 mL TBS.
7. Isolate beads, discard supernatant, and wash briefly by inversion with 1 mL Tris (see **Note 12**).
8. Resuspend beads in 100 μ L Tris.

3.6 Isolation of Peptides for Mass Spectrometry

1. Remove 5 μ L (5%) of bead suspension and transfer to a 1.5 mL tube with an O-ring gasket; store at 4 °C (see **Note 13** and Subheadings 2.8 and 3.7).
2. With the remaining 95% sample, isolate beads, discard supernatant, and resuspend in 100 μ L ammonium bicarbonate.
3. Isolate beads, discard supernatant, and resuspend in 100 μ L of 10 mM dithiothreitol (see **Note 14**).
4. Incubate beads in dithiothreitol for 30 min at 50 °C.
5. Cool samples to room temperature, isolate beads, and discard supernatant.
6. Resuspend beads in 100 μ L of freshly made iodoacetamide and incubate in the dark for 30 min (see **Note 15**).
7. Isolate beads and discard supernatant.
8. Wash beads in 100 μ L ammonium bicarbonate, mixing by gentle inversion.
9. Resuspend beads in 100 μ L ammonium bicarbonate.
10. Transfer bead suspension to an Eppendorf Protein LoBind tube.
11. Add 1 μ g trypsin. To prepare trypsin stock, dilute vial of lyophilized trypsin (20 μ g in vial) in 40 μ L of the provided resuspension buffer to make 0.5 μ g/ μ L (see **Note 16**).
12. Incubate at 37 °C for 16–18 h.
13. Add 2 μ L formic acid to stop the trypsin enzyme reaction.
14. Isolate beads and transfer supernatant (tryptic peptides) to a new Eppendorf Protein LoBind tube.
15. Store tryptic peptides at –80 °C until ready to send to for mass spectrometry.

3.7 Check Samples for Mass Spectrometry

1. Isolate beads from the 5% sample held back in Subheading 3.6, **step 1** and discard supernatant.
2. Elute beads in 20 μ L elution buffer by boiling for 5 min.
3. Isolate beads, transfer supernatant to a new tube, and discard beads.
4. Add 5 μ L pink glycerol to samples.
5. Load entire sample for western blot.
6. Block membrane in milk solution for 30 min.
7. Incubate with streptavidin-HRP antibody for 45 min.
8. Wash membrane in generous amounts of TBS-T for at least 5×20 min.
9. Add peroxide-based chemiluminescent reagent and image (Fig. 2).

4 Notes

1. When sending samples for mass spectrometry, a new batch of biotin stock solution should be made.
2. To achieve a 10 M solution of urea, low heat (no more than 30 °C) must be used with stirring for 30–60 min. If the temperature of the urea gets exceeds 37 °C, it can break down.
3. The mixed bead resin turns from a blue/green color to orange/gold when it has reached its deionization capacity. If a majority of the beads turn orange/gold, add more resin in 10 g increments and continue stirring. The appropriate amount of mixed bead resin has been added when approximately 50% of the beads remain blue/green in color.
4. It is strongly suggested that only these beads be used for mass spectrometry. These beads have a uniform surface which is essential to reduce non-specific binding and prevent noise in the mass spectrometry results. The use of these beads is especially applicable given the complex, whole cell fraction of epidermal culture samples in urea sample buffer. Streptavidin-conjugated agarose beads should not be used for downstream mass spectrometry.
5. Stock solutions of 100 mM dithiothreitol prepared in 100 mM ammonium bicarbonate can be aliquoted and stored at -80 °C. For use, thaw and dilute to 10 mM in ammonium bicarbonate. Do not refreeze or reuse dithiothreitol that has been thawed.
6. Iodoacetamide should be store at 4 °C with desiccation. Bring to room temperature before opening bottle. Make fresh for each use.
7. An empty vector or expression of an unfused BirA* can be used as the control for BioID in 2D and 3D epidermal keratinocyte cultures. However, we found that expression of the BirA* biotin ligase alone is lost upon epidermal differentiation

(Fig. 3). As such, efforts for molecular cloning of BirA* into an expression vector may not be worthwhile.

8. The protein concentration of samples in urea sample buffer should be between 4 and 5 µg/µL to allow for sufficient dilution of the samples in subsequent steps. Specifically, the sodium dodecyl sulfate needs to be diluted to <0.5% to prevent precipitation at 4 °C. For 2D cultures, three 10 cm dishes of confluent keratinocytes ($>15 \times 10^6$ total cells) can be lysed in 3 mL of urea sample buffer. This will provide plenty of samples and can be scaled down but can also be used for optimization. For 3D cultures based on a 12-well format, 2–4 cultures should be combined for earlier time points (days 1–5). Later time points (days 6–12) need 2–3 cultures to provide sufficient material, lysed in 3 mL urea sample buffer.
9. The cell lysate in urea sample buffer is viscous because of the DNA in solution. The viscosity is alleviated by shearing the DNA through a 27 gauge needle. Urea sample buffer solubilizes all cellular components, so there is no need to centrifuge, boil, or further manipulate the samples. Use of urea sample buffer eliminates the need for sample sonication, a common source of inter-experimental variability. Due to the content of glycerol and the pyronin Y dye, samples can be directly loaded into gels for electrophoresis.
10. Due to the composition of urea sample buffer, many commercial colorimetric protein assays are not suitable.
11. After streptavidin precipitation, samples can also be used to perform benchtop BioID to validate the BirA* fusion protein or to use a candidate approach to identify interacting proteins (Fig. 4). For this approach, samples would be eluted in elution buffer (as in Subheading 2.8, **step 1**) and run in a conventional Western blot protocol. For benchtop BioID, streptavidin-conjugated agarose beads are suitable for use.
12. These final washes serve to remove polymers derived from the detergents and ions derived from the salts which can greatly interfere with the mass spectrometer.
13. Before sending samples for mass spectrometry, it is advisable to check the controls for low biotinylation and BirA*-fusion protein samples for high biotinylation.
14. Dithiothreitol reduces any remaining disulfide bonds in the biotinylated proteins bound to the streptavidin to allow for more efficient trypsin digestion.
15. Iodoacetamide caps the reduced cysteine residues to inhibit their reactivity and ability to form cross-links.
16. Aliquot any unused trypsin by 5 µL and store at –80 °C. Do not refreeze once thawed.

Acknowledgment

This work was supported by a NIH grant AR072773 and a Dermatology Foundation Career Development Award grant to B.E.P.W., and a NIH grant AR062110 to S.G. Work was performed with the support of the Northwestern University Skin Disease Research Center funded by NIH grant AR057216 and Northwestern Proteomics funded by NIH grants CA060553 and GM108569.

References

1. Blanpain C, Fuchs E (2009) Epidermal homeostasis: a balancing act of stem cells in the skin. *Nat Rev Mol Cell Biol* 10 (3):207–217. 10.1038/nrm2636 [PubMed: 19209183]
2. Koster MI, Roop DR (2007) Mechanisms regulating epithelial stratification. *Annu Rev Cell Dev Biol* 23:93–113. 10.1146/annurev.cellbio.23.090506.123357 [PubMed: 17489688]
3. Fuchs E (2016) Epithelial skin biology: three decades of developmental biology, a hundred questions answered and a thousand new ones to address. *Curr Top Dev Biol* 116:357–374. 10.1016/bs.ctdb.2015.11.033 [PubMed: 26970628]
4. Bliss E, Heywood WE, Benatti M et al. (2016) An optimized method for the proteomic profiling of full thickness human skin. *Biol Proced Online* 18(15). 10.1186/s12575-016-0045-y
5. Mikesch LM, Aramadhaka LR, Moskaluk C et al. (2013) Proteomic anatomy of human skin. *J Proteomics* 12(84):190–200. 10.1016/j.jprot.2013.03.019
6. Rice RH, Durbin-Johnson BP, Mann SM et al. (2018) Corneocyte proteomics: applications to skin biology and dermatology. *Exp Dermatol* 27(8):931–938. 10.1111/exd.13756 [PubMed: 30033667]
7. Arnette C, Koetsier JL, Hoover P et al. (2016) In vitro model of the epidermis: connecting protein function to 3D structure. *Methods Enzymol* 569:287–308. 10.1016/bs.mie.2015.07.015 [PubMed: 26778564]
8. Eyerich K, Brown SJ, Perez White BE et al. (2019) Human and computational models of atopic dermatitis: a review and perspectives by an expert panel of the International Eczema Council. *J Allergy Clin Immunol* 143 (1):36–45. 10.1016/j.jaci.2018.10.033 [PubMed: 30414395]
9. Roux KJ, Kim DI, Raida M, Burke B (2012) A promiscuous biotin ligase fusion protein identifies proximal and interacting proteins in mammalian cells. *J Cell Biol* 196:801–810. 10.1083/jcb.201112098 [PubMed: 22412018]
10. Roux KJ, Kim DI, Burke B (2013) BioID: a screen for protein-protein interactions. *Curr Protoc Protein Sci* 74:19.23.1–19.23.14. 10.1002/cpps.51 [PubMed: 24510646]
11. Perez White BE, Ventrella R, Kaplan N et al. (2017) EphA2 proteomics in human keratinocytes reveals a novel association with afadin and epidermal tight junctions. *J Cell Sci* 130:111–118. 10.1242/jcs.188169 [PubMed: 27815408]
12. Lee HJ, Hota PK, Chugha P et al. (2012) NMR structure of a heterodimeric SAM:SAM complex: characterization and manipulation of EphA2 binding reveal new cellular functions of SHIP2. *Structure* 20:41–55. 10.1016/j.str.2011.11.013

Fig. 1.

Expression of EphA2-BirA* fusion protein in 2D and 3D primary epidermal keratinocyte cultures detected by HA-tag (green) and distribution of biotinylated proteins as shown by streptavidin reactivity (red). In 2D cultures, immunofluorescence was performed after 24 h in 1.2 mM $[Ca^{2+}]$ to initiate epidermal differentiation and then an additional 24-h exposure to biotin in 1.2 mM $[Ca^{2+}]$. Immunostaining was performed after 72 h (days 6–9 after lifting to the air-liquid interface) exposure to biotin in 3D cultures. Expression of the EphA2-BirA* fusion protein and biotin-conjugating activity localized throughout the cells in 2D culture but was predominant at cell borders. In 3D cultures, the fusion protein expression and biotinylating activity were restricted to the cell periphery. The localization pattern of the EphA2-BirA* fusion protein mirrors that of the endogenous protein in 2D and 3D cultures [10]. Scale bars: 20 μ m. DAPI was used to counterstain the nucleus

Fig. 2. Detection of biotinylated proteins following streptavidin precipitation in 3D skin cultures. A small fraction (5%) of the streptavidin-IP samples from 3D skin cultures expressing an empty vector (EV) control or an EGFR-BirA* fusion protein was run by conventional western blot and incubated with a horseradish peroxidase-conjugated streptavidin antibody. There is minimal detection of biotinylated proteins in the control and high streptavidin reactivity in the sample from EGFR-BirA*. *MWM* molecular weight marker

Fig. 3.

Expression pattern of the BirA* protein in primary keratinocyte 2D cultures. The BirA* bacterial biotin ligase was expressed at low levels in proliferating keratinocytes (0.07 mM $[\text{Ca}^{2+}]$ with growth supplements) and exhibited minimal protein biotinylation when exposed to biotin for 24 h. After induction of epidermal differentiation for 24 h in 1.2 mM $[\text{Ca}^{2+}]$ and then biotin exposure for an additional 24 h, the expression and biotin ligase activity of BirA* were lost

Fig. 4.

Benchtop BioID analysis in 2D epidermal cultures. Benchtop BioID can be used to validate BirA* fusion proteins or to perform a candidate-based approach to identifying putative interactors. The EphA2-BirA* fusion protein biotinylated itself, as expected, and the known EphA2 interactor SHIP2 [12]. EphA2-BirA* did not biotinylate ephrin-A1 as the biotin ligase was cloned in the cytoplasmic tail and ephrin-A1 interacts with EphA2 in extracellular space. GAPDH was also not biotinylated. *, EphA2-BirA* fusion protein