SUPPLEMENTARY MATERIALS:

SUPPLEMENTARY METHODS

Generation of GFP*topaz*-collagen and mCherry-collagen Imaging Probes and pGL3-puro selection vector

To generate probes for imaging type I collagen, our strategy was to generate cDNA constructs consisting of GFP*topaz* (hereafter referred to as GFP*tpz*), fused to the *Col1a2* sequence. By targeting the proα2(I) chain, each collagen trimer should theoretically contain only a single GFP moiety, since there is one α2(I) chain per trimer. We reasoned this would be less disruptive than targeting the proα1(I) chain, which could result in trimers containing two GFP moieties or potentially three, since proα1(I) chains can form homotrimers[1].

Three different GFP*tpz*-α2(I)-collagen constructs were initially engineered (see Fig. 1 in main manuscript). In construct A, the GFP*tpz* tag replaced the N-propeptide and N-telopeptide. In construct B, GFP*tpz* replaced the N-propeptide, but the N-telopeptide was left in frame after GFP. Since the cloning strategy for constructs A and B removes the N-terminal propeptide and its cleavage site, the GFP moiety should theoretically not be cleaved off during collagen processing. Construct C was generated as a control in which GFP was inserted in front of the N-terminal propeptide. Therefore, the GFP should be cleaved off during procollagen processing and should not be found in assembled collagen fibrils. The GFP*tpz* coding sequence was obtained from Packard Instrument Co., Meriden, CT, USA (now licensed to Clontech, Mountainview, CA). Isolation of the mouse cDNA for *Col1a2* has been described previously[2]. The plasmid containing this cDNA (pMSCV-puro-*Col1a2*) was used as a template for cloning by PCR amplification and the amplified constructs were cloned into the pcDNA3 vector (Invitrogen Corporation, Carlsbad, CA), which has a CMV promoter.

Two modified versions of construct A were next generated. The first was an mCherry version of construct A in which the mCherry red fluorescent protein (Clontech) was inserted in place of GFP*tpz*. This mCherry construct was named construct D. The second was a modified version of construct A in which expression of the GFP*tpz*-collagen fusion construct was placed under control of a 3.6kb fragment of the *Col1a1* promoter (provided by Dr. Barbara Kream, University of Connecticut Health Center) instead of the CMV promoter used in construct A. This new construct was termed construct E. Sequences of all the constructs were verified by DNA sequencing. For a schematic diagram of all the expression constructs A through E, see Fig. 1 in main manuscript. Detailed information on the cloning steps for generation of these constructs is provided below. Please also see supplementary Figs. S1-S4 and supplementary table S1.

Details of Cloning Steps for Generation of GFP*tpz*-collagen and mCherry-collagen Constructs

(i) Generation of GFPtpz-α2(I)-collagen Construct A (#142-8):

Construct A was generated in three steps (see supplementary Fig. S1 for a schematic diagram of the cloning strategy):

Step 1 - An intermediate construct IA was generated containing the murine *Col1a2* N-terminal cDNA with deletion of the N-propeptide and N-telopeptide coding sequence. This was generated by two sequential PCR amplifications. The first PCR used the primers Col2-FP5 and Col2-RP (see Table S1), with pMSCV-puro-*Col1a2* (#128) as a template. This plasmid contained the mouse *Col1a2* cDNA[2]. The second PCR used the Col2-FP2 forward primer and the same reverse primer Col2-RP (see Table S1), using the first PCR product as a template. The second PCR products were digested with EcoRI and NotI then cloned into the EcoRI and NotI sites of the pcDNA3 vector (Invitrogen) to generate construct IA (#139-6: *Col1a2* N terminal fragment with deletion of the N-propeptide and N-telopeptide coding sequence). In this construct, an EcoRV site was introduced between the coding sequences for the signal peptide and mature chain for subsequent insertion of the GFP-*tpz* cDNA. Construct IA was confirmed by DNA sequencing.

Step 2 - The GFP-*tpz* cDNA was released from Construct ID (see below) with EcoRV, and subcloned into the EcoRV site of Construct IA (#139-6: N terminal fragment with deletion of N-propeptide and N-telopeptide coding sequence) to give rise to the second intermediate Construct IB (#140-5), in which the

cDNA coding for the N-propeptide and N-telopeptide was replaced by the GFP-*tpz* cDNA. The orientation of the GFP-cDNA was verified by PCR with the primers, Col2-FP2 and Col2-GFPR (see Table S1).

Step 3 - The m*Col1a2*-N-GFP-*tpz*, released from Construct IB by digestion with EcoRI and XhoI, and the *Col1a2* C-terminal cDNA, released from pMSCV-puro-*Col1a2* (#128) with XhoI and NotI were ligated into the EcoRI and NotI sites of the pcDNA3 vector (Invitrogen) to generate the final Construct A.

(ii) Generation of GFPtpz-α2(I)-collagen Construct B (#141-7):

Construct B was also generated in three steps (see Fig. S2 for a schematic diagram of the cloning strategy)

Step 1 - An intermediate construct IC was generated containing the *Col1a2* N-terminal cDNA with deletion of the N-propeptide coding sequence. This *Col1a2* N-terminal cDNA was generated by two sets of PCR reactions. The first PCR used the primers Col2-FP1 and Col2-RP (see Table S1), with pMSCV-puro-*Col1a2* as a template; the second PCR used the Col2-FP2 forward primer and the same reverse primer Col2-RP (see Table S1) with the first PCR product as a template. The second PCR products were cut with EcoRI and NotI, and then cloned into the EcoRI and NotI sites of the pcDNA3 vector (Invitrogen) to generate Construct IC (#132-8, N terminal fragment with deletion of N-propeptide). In this construct, an EcoRV site was introduced between the coding sequences for the signal peptide and mature chain for insertion of the GFP-*tpz* cDNA. Construct IC was confirmed by DNA sequencing.

Step 2- The GFP*tpz* cDNA was amplified by PCR using the pBC-KS vector containing GFP*tpz* cDNA (#206,) as a template and the primers, Col2-GFPF and Col2-GFPR (see Table S1). The PCR products were cut with EcoRV and then cloned into the EcoRV site of Construct IC to generate the second intermediate Construct ID (#133-2), containing the *Col1a2* N-terminal cDNA with N-propeptide coding sequence replaced by GFP*tpz* cDNA. The orientation of the GFP*tpz* cDNA was confirmed by PCR screening with the primers, GFP-F and Col2-SRP (see table S1). Construct ID was confirmed by DNA sequencing.

Step 3- the final Construct B (#141-7) was generated by subcloning the *Col1a2*-N-GFP*tpz* fragment, released from Construct ID by EcoRI and XhoI, and the *Col1a2* C-terminal cDNA released from pMSCV-puro-*Col1a2* by XhoI and NotI into the EcoRI and NotI sites of pcDNA3 vector.

(iii) Generation of GFPtpz-α2(I)-collagen Construct C (#150-6):

Construct C was generated in three steps (see Fig. S3 for a schematic diagram of the cloning strategy)

Step 1 - Two rounds of PCR reactions were used to introduce an EcoRV cut site between the coding sequences for the signal peptide and N-terminal propeptide of *Col1a2*. The first PCR used primers Col2-FP3 and Col2-RP (see Table S1) with pMSCV-puro-*Col1a2* as a template, and the second PCR used the Col2-FP2 forward primer and the same reverse primer Col2-RP (see Table S1) with the first PCR product as a template. Three clones were sequenced and all had mutations. However one of the clones (termed IE^{Adel}, #138-12) had the mutation in the signal peptide region so this mutated signal peptide coding sequence was replaced by subcloning the N-terminal *Col1a2* cDNA, released by EcoRV and XhoI into the EcoRV and XhoI sites of Construct ID to generate the intermediate Construct IE (#143-2), thus creating an EcoRV site between the coding sequence for the signal peptide and N-terminal propeptide.

Step 2 - The GFP*tpz* cDNA was released from Construct ID with EcoRV, and subcloned into the EcoRV site of Construct IE, to produce the intermediate Construct IF (#146-8), in which the GFP*tpz* cDNA was inserted between the coding sequence for the signal peptide and N-propeptide). The orientation of the GFP*tpz* cDNA was confirmed by PCR screening with the primers Col2-GFPF and Col2-SRP (see Table S1).

Step 3 - The final Construct C (#150-6) was generated by subcloning the m*Col1a2*-N-Ins-GFP-*tpz* cDNA released from Construct IF (#146-8) by digestion with EcoRI and XhoI, and the C-terminal *Col1a2* cDNA released from pMSCV-puro-*Col1a2* with XhoI and NotI into the EcoRI and NotI sites of the pcDNA3 vector.

(iv) Generation of mCherry-α2(I)-collagen Construct D (#154-11):
A single cloning step was used to generate the mCherry-α2(I)-collagen construct from construct A. First, the mCherry cDNA was amplified by the primers Cherry-FP and Cherry-RP (see Table S1), using the pmcherry-N1 vector as a template (Clontech). The PCR products were cut with EcoRV, and cloned into Construct A (#142-8) to replace the GFP*tpz* cDNA, thus giving rise to the mCherry-α2(I)-collagen Construct D (#154-11, *Col1a2* cDNA with N-propeptide and N-telopeptide replaced by mCherry cDNA).

(v) Generation of GFPtpz-α2(I)-collagen Construct E (RD078-10):
Construct E was generated in two steps (see Fig. S4 for a schematic diagram of the cloning strategy)

Step1 – The C-terminal end of the *Col1a2* cDNA, including the C-terminal non-coding region, was obtained by PCR with the primers Col1a2-AU1 and Col1a2-AL1 (see Table S1), using mouse genomic DNA as a template. The PCR product was subsequently cloned into the EcoRI and XhoI sites of pBluescript-SK vector (Stratagene, La Jolla, CA), to give rise to pSK-3' noncoding region (RD065-6).

Step 2 – Construct E (RD078-10) was generated by ligating the HindIII/NheI fragment from construct A (#142-8), blunted at the HindIII end, the NheI/EcoRI fragment (from #142-8) and the EcoRI/XhoI fragment (from RD065-6) into the EcoRV and SalI sites of pBC-KS vector (kindly provided by Dr. Barbara Kream, University of Connecticut Medical Center), downstream of the 3.6kb type I collagen promoter. The sequence around the two junctions was confirmed by sequencing with the primers Col1a2-CPU and Col1a2-CPL (see Table S1).

(vi) Generation of puromycin selection vector pGL3-puro:
The puromycin expressing vector (pGL3-puro) was generated by subcloning the fragment containing promoter-puro cDNA from the pMSCV-puro-*Col1a2* vector (see above) into the pGL3-basic vector (Promega Corporation, Madison, WI) to replace the luciferase gene. The pGL3-puro vector was then used for co-transfection with GFP*tpz*-collagen and mCherry-collagen constructs for generation of stable cell lines.

All PCR amplifications were performed using high fidelity Deep Vent DNA polymerase (New England Biolabs), and constructs and intermediate constructs confirmed by sequencing. Table S1 lists the primers used and the enzyme cut sites are also indicated in bold font for each primer.

MLO-colGFP and MLO-colCherry Co-culture, Parachute and Interface Cultures
To examine whether individual osteoblasts assemble their own collagen fibers or multiple cells cooperate to assemble collagen. Three types of co-culture experiments were set up (see Fig. 7). To co-culture the cells at a 1:1 ratio, MLO-colGFP and MLO-colCherry cells were plated into collagen coated lab-tek chamber slides with each cell line plated at 1 x 10^4 cells/cm^2 (total number of cells combined is 2 x 10^4 cells/cm^2). At confluence, fresh media was added containing 100µg/ml L-ascorbic acid phosphate magnesium salt n-hydrate and the cells were cultured for a further 6 days to allow collagen deposition before fixation and fluorescence imaging. For "parachute" experiments, MLO-colGFP cells were plated in collagen coated lab-tek chamber slides at 2 x 10^4 cells/cm^2. At confluence, fresh media was added containing 100µg/ml L-ascorbic acid phosphate magnesium salt n-hydrate and the cells were cultured for a further 3 days to allow initiation of collagen assembly. MLO-colCherry cells were then seeded onto the MLO-colGFP cell layer at low densities of 100-500cells/cm^2 to allow visualization of mCherry-collagen networks assembled by individual cells or isolated small groups of cells. For "interface" experiments, the MLO-colGFP or MLO-colCherry cells were plated separately at high density (1.7 x 10^6 cells/ml) in 25µl droplets of growth media, positioned about 1-2mm apart on collagen coated lab-tek chamber slides. The cells in the droplets were allowed to adhere to the slide for 2h at 37^{o}C in a humidified 5%CO_2 incubator to form droplet-sized colonies and then fresh media containing 100µg/ml L-ascorbic acid phosphate magnesium salt n-hydrate was added to the wells. The separated colonies of MLO-colCherry and MLO-colGFP cells were then allowed to grow and expand towards each other over 4-6 days until interfaces were formed between the colonies of red and green collagen expressing cells. In all three formats, if multiple cells co-operate to assemble collagen, we would expect to see fibers containing both red and green

collagen. However, if individual cells assemble their own collagen fibers without contributions from other cells, we would expect separate patches of green or red fibers, but not fibers containing both red and green collagen.

Immunogold staining and Transmission Electron Microscopy

For immunogold staining, cells were grown on thermanox coverslips (Nalge Nunc/Thermo Fisher Scientific) in 24 well plates in 1ml media and immunogold labeling was performed on viable cultures without prior fixation as described previously[3]. All antibodies were diluted in culture media to maintain cell viability, with additional supplements including 20mM HEPES, 30µg/ml gentamycin and 25µg/ml aprotinin. The 10% FBS present in the culture media provides adequate background blocking for antibody staining and no additional background blocking step was done. The coverslips were rinsed in PBS, then floated cell side down in 50µl primary antibody, diluted in culture medium as above. The samples were incubated for 2hr at room temperature, washed 6X in PBS and floated cell side down on 50µl secondary antibody -6nm or -12nm gold conjugate (Jackson Immunoresearch), diluted 1:10 in culture medium. They were incubated for 2hr at room temperature and rinsed 6X in PBS then rapidly once in 0.1M sodium cacodylate buffer (pH 7.4), before fixation for 60 min in 2.5% glutaraldehyde in 0.1M cacodylate buffer containing 0.05% tannic acid. After fixation, the samples were washed 3X in 0.1M cacodylate buffer at 4^{O}C and post fixed with 1% osmium tetroxide for 1.5h. Dehydration was performed through graded ethanols and specimens were embedded in epoxy resin (Embed-812, Electron Microscopy Sciences, Hatfield, PA). Ultrathin sections were cut on an EM UC7 ultramicrotome (Leica Microsystems Inc., Buffalo Grove, IL), stained with uranyl acetate and lead citrate and observed with a CM12 electron microscope (FEI, Hillsboro, OR) at 80 kV accelerating voltage.

Procedure for Sequential Extraction of Collagen from the Cell Layer

For the sequential extraction of collagen, all procedures were performed on ice. The cell layer was washed twice with ice cold PBS and then extracted with 5ml salt extraction buffer per 150mm dish, consisting of 0.05M Tris, pH 7.5, 1M NaCl containing N-Ethylmaleimide (NEM) (0.5ml of 67mM stock in PBS/0.3M EDTA) plus sigma protease inhibitor cocktail (cat#P8340).The cells and extracellular matrix were dislodged into the buffer using a cell scraper and the suspension was transferred to a 15ml tube along with a wash of 3ml salt extraction buffer. Additional protease inhibitors were immediately added as follows: phenylmethylsulfonyl fluoride (PMSF) (80µl of 100mM stock in EtOH), pepstatin-A (32µl of 1mg/ml stock in 95% EtOH). The sample was incubated overnight on an end-over rotator then centrifuged at 15,000g for 30 min at 4^{O}C. The supernatant was used as the salt extract. The pellet was then washed in 10ml ice cold PBS and resuspended in 8ml 0.5M acetic acid containing the protease inhibitor NEM as described above. PMSF and pepstatin-A were immediately added as described above. The sample was then extracted for 24h at 4^{O}C on an end-over rotator and centrifuged at 15,000g for 30 min at 4^{O}C. The supernatant was used as the acid soluble collagen extract. The pellet was washed twice in 10ml ice cold PBS then resuspended in 5ml 0.5M acetic acid containing 1mg/ml pepsin (Sigma-Aldrich). The sample was digested overnight at 4^{O}C on an end-over rotator and the reaction was stopped by adding 250µl pepstatin A stock. The sample was centrifuged at 15,000g for 30 min at 4^{O}C and the supernatant was used as the pepsin soluble collagen extract.

Immunoprecipitation Procedure

Immunoprecipitation was performed using a Pierce Classic IP kit (Pierce/Thermo Fisher Scientific.), with all steps performed on ice or at 4^{0}C. 600µl media samples were used for immunoprecipitation and Tris pH 7.4 was added to a final concentration of 50mM to maintain stable pH. For salt, acid or pepsin extracted samples a 30kDa MWCO microcon centrifugal filter unit (EMD Millipore, Billerica, MA) was used to exchange the buffer to IP lysis/wash buffer prior to immunoprecipitation. The samples were first precleared by incubation with 6µl normal rabbit serum plus 20µl protein A/G agarose for 30 min on an end-over rotator at 4^{0}C. The samples were then spun through spin columns and the filtrate (precleared sample) was used for immunoprecipitation. Precleared samples (600µl) were incubated overnight on an end-over rotator at 4^{O}C with 6µl rabbit polyclonal antiserum against GFP (to precipitate the GFP*tpz*-tagged construct) or against DsRed (to precipitate the mCherry construct). Control samples were incubated with 6µl normal rabbit serum to confirm specificity. Immune complexes were precipitated by addition of 20µl protein A/G agarose and incubation for 2h at 4^{O}C. The sample was then spun through a fresh spin column and the

protein A/G immunoprecipitate above the column was washed 3 times with 200μl wash buffer, then once with conditioning buffer. Immunoprecipitated proteins were eluted by adding 20μl 1X Laemmli (reducing) sample buffer to the protein A/G agarose and heating at 100^{O}C for 5 minutes, followed by centrifugation of the spin column to recover the eluted proteins.

REFERENCES FOR SUPPLEMENTARY METHODS

1. Jimenez SA, Bashey RI, Benditt M, Yankowski R. Identification of collagen alpha1(I) trimer in embryonic chick tendons and calvaria. *Biochem Biophys Res Commun* 1977 Oct 24; **78**(4)**:** 1354-1361.
2. Phillips CL, Morgan AL, Lever LW, Wenstrup RJ. Sequence analysis of a full-length cDNA for the murine pro alpha 2(I) collagen chain: comparison of the derived primary structure with human pro alpha 2(I) collagen. *Genomics* 1992 Aug; **13**(4)**:** 1345-1346.
3. Dallas SL, Keene DR, Bruder SP, Saharinen J, Sakai LY, Mundy GR*, et al.* Role of the latent transforming growth factor beta binding protein 1 in fibrillin-containing microfibrils in bone cells in vitro and in vivo. *Journal of Bone & Mineral Research* 2000; **15**(1)**:** 68-81.

Table S1: Primers Used for Generation of the GFPtpz-collagen and mCherry-collagen Constructs

Primer Name	Primer sequence (5'-3')
Col2-GFPF	**EcoRV** GCC**GATATC**GTGAGCAAGGGCGAGGAGCTGT
Col2-GFPR	**EcoRV** GCG**GATATC**CTTGTACAGCTCGTCCATGCCGAGAGTG
GFP-F	GTCGCTAGCATGGTGAGCAAGGGCGAGGAGCTGT
Col2-FP1	**EcoRV** GCTGCTTGCAGTAACTTCGTGCCTAGCAACATGCCAA**GATATC** TATTCTGACAAAGGAGTTTCAT
Col2-FP2	**EcoRI** GTC**GAATTC**GTAGACATGCTCAGCTTTGTGGATACGCGGACTC TGTTGCTGCTTGCAGTAACTTCG
Col2-FP3	**EcoRV** GCTGCTTGCAGTAACTTCGTGCCTAGCAACATGCCAA**GATATC** TATTTGCAATCGGGATCAGTAC
Col2-FP5	**EcoRV** GCTGCTTGCAGTAACTTCGTGCCTAGCAACATGCCAA**GATATC** GGACCAATGGGTTTAATGGG
Col2-RP	CCAGGGCCAGATGAAACTC
Col2-SRP	CCAGGGCCAGATGAAACTC
Cherry-FP	**EcoRV** CC**GATATC**GTGAGCAAGGGCGAGGAGGATAAC
Cherry-RP	**EcoRV** GA**GATATC**CTTGTACAGCTCGTCCATGCCG
Col1a2-AU1	GAATGGGGCAAGACAATCATTG
Col1a2-AL1	**XhoI** **SacII** GTT**CTCGAGCCGCGG**TGGCTGGTAGAGATGCAGATTC
Col1a2-CPU	ACCCCAGCGAAGAACTCATAC
Col1a2-CPL	GGGCCAAGGGAGGAGACTTAG

Figure S1: Cloning strategy for GFP*tpz*-α2(I)-collagen construct A

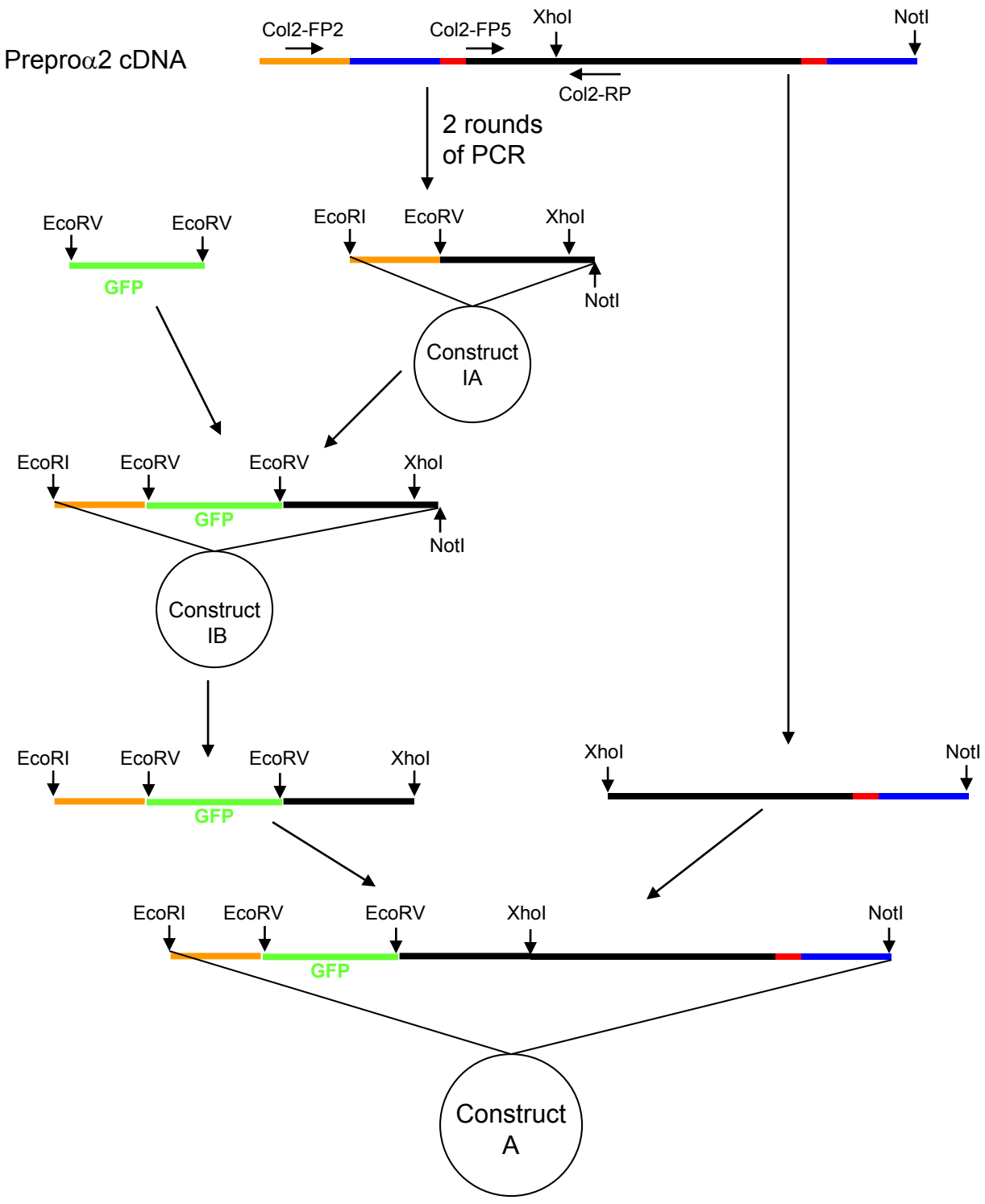


Figure S2: Cloning strategy for GFP*tpz*-α2(I)-collagen construct B

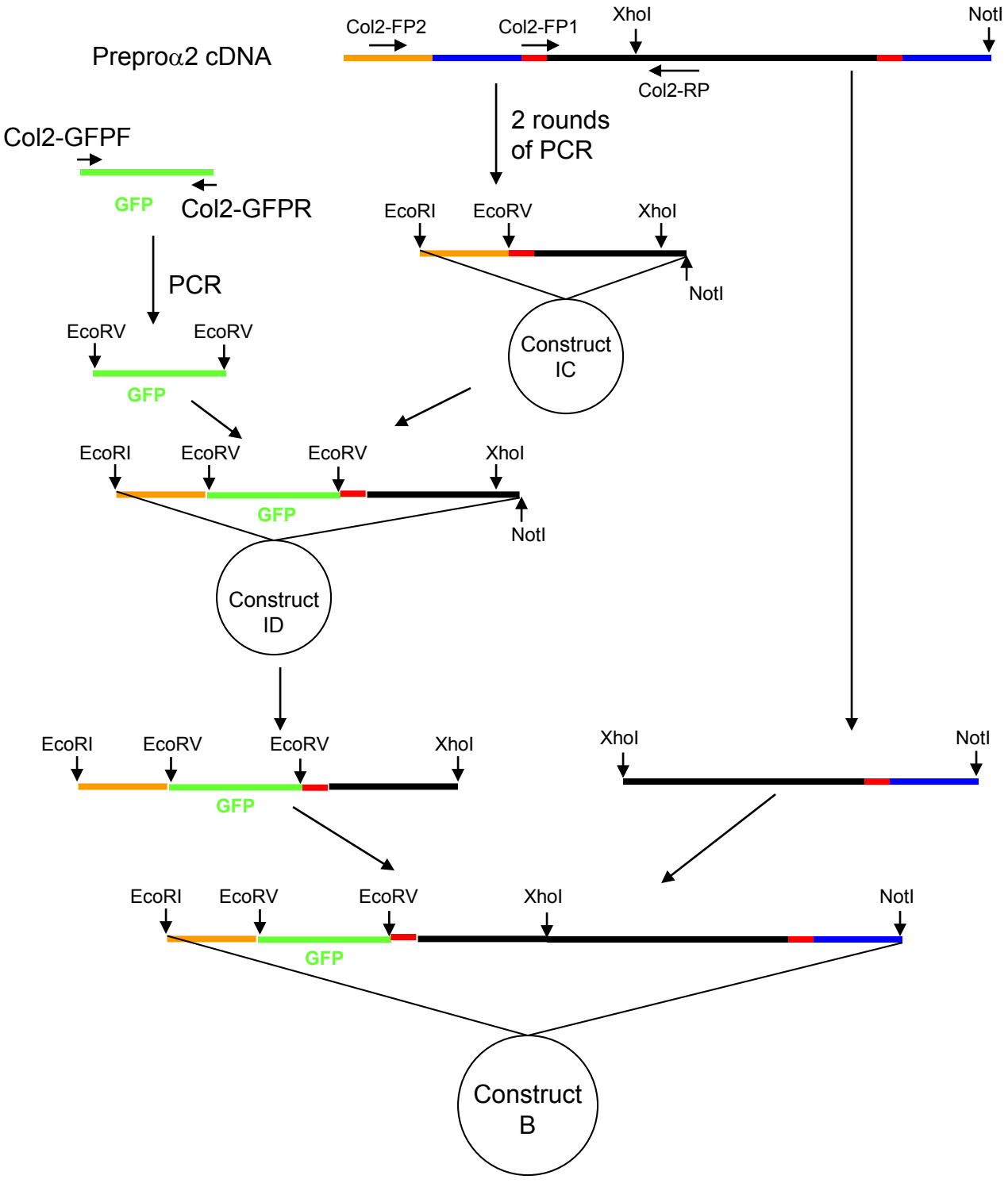


Figure S3: Cloning strategy for GFP*tpz*-α2(I)-collagen construct C

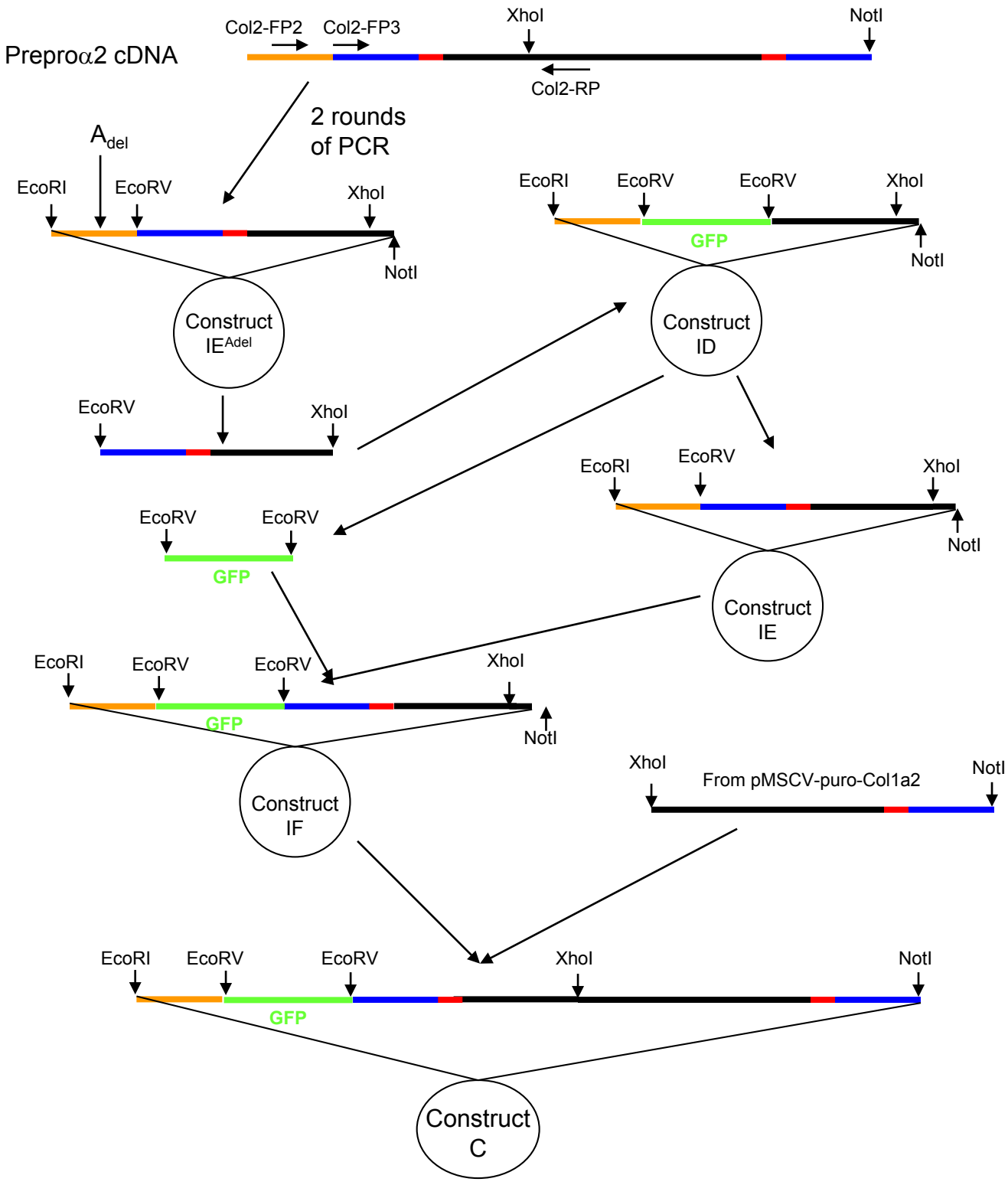


Figure S4: Cloning strategy for GFP*tpz*-α2(I)-collagen construct E

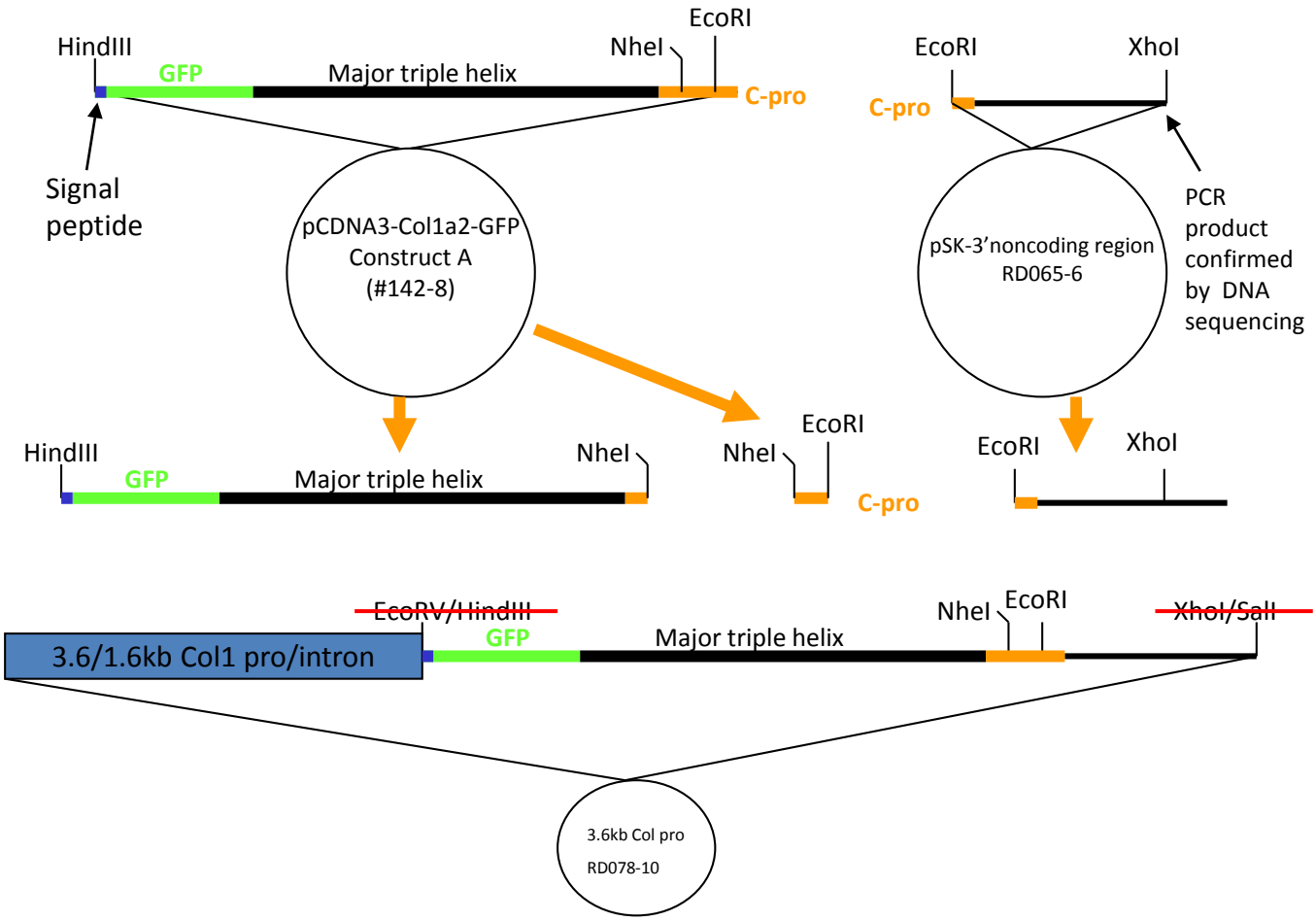


Figure S5

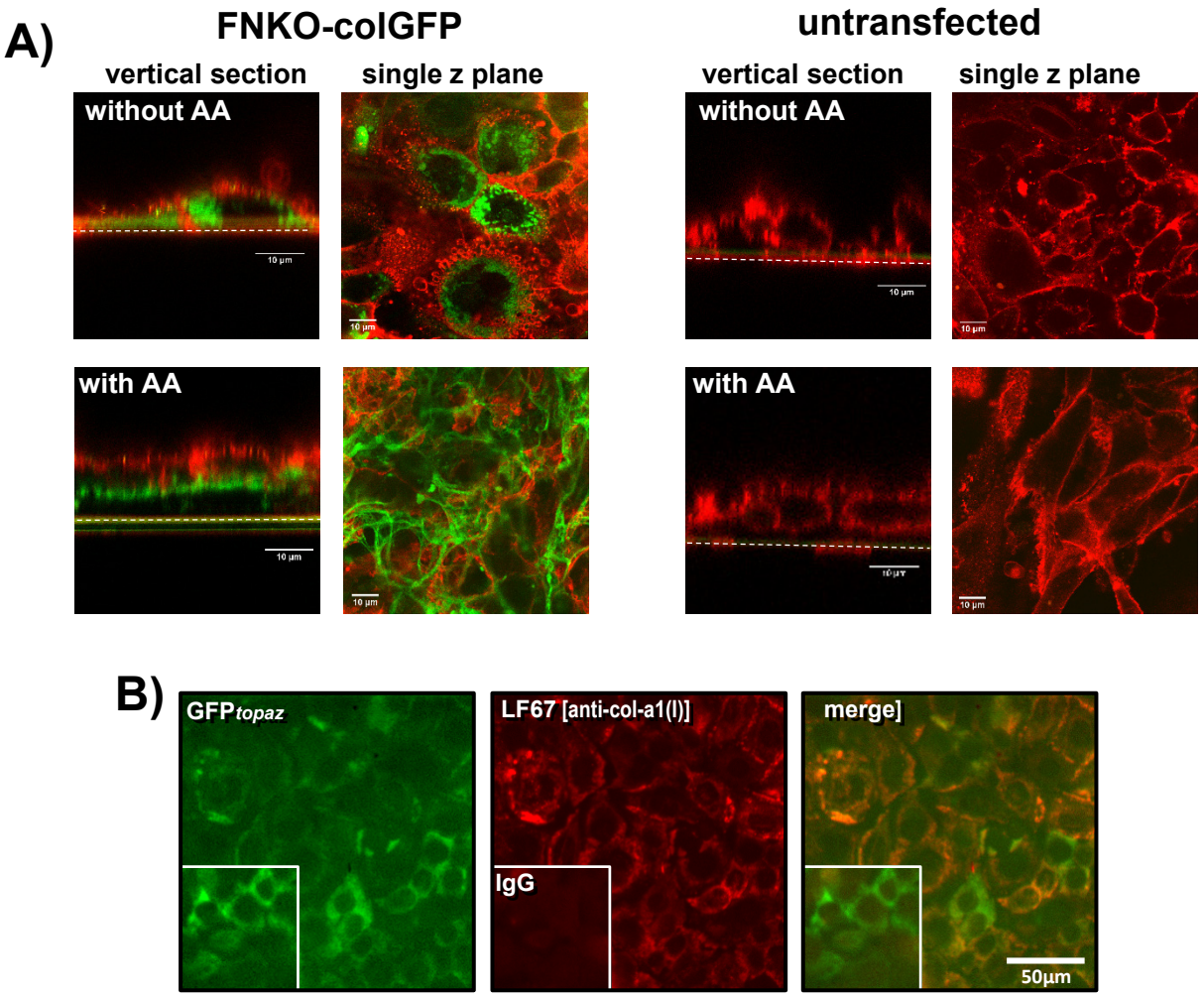
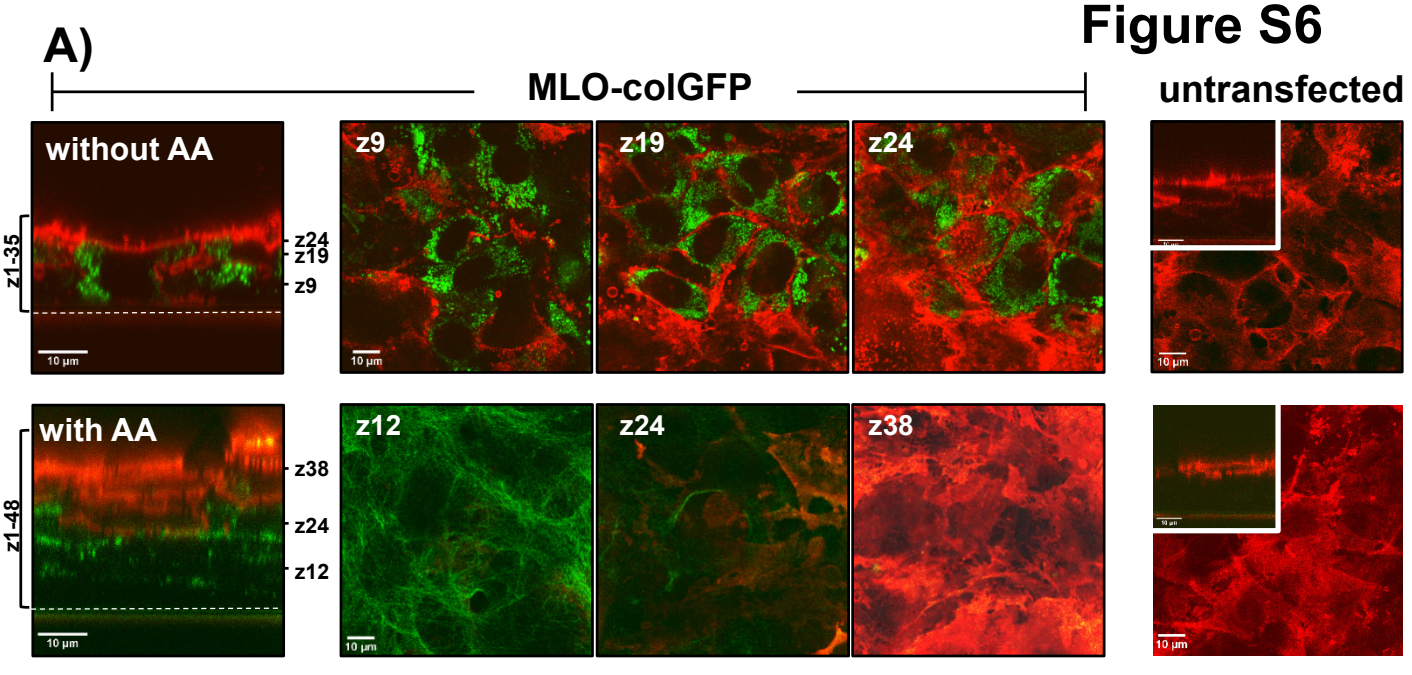


Fig. S5: GFP*tpz* Collagen is Intracellular in FNKO-colGFP cells in the Absence of Ascorbate – A) Confocal vertical section and individual Z slice through FNKO-colGFP and untransfected control FN-null-MEF cells cultured without ascorbate (upper panels) or with ascorbate (lower panels) and stained with the membrane dye DiD (red). Note that without ascorbate the GFP*tpz*-collagen signal (green) remains intracellular (i.e. within the boundaries of the cell membranes). In contrast, with ascorbate, the GFP*tpz*-collagen signal is localized in extracellular fibrils, which are predominantly deposited below the cell layer and there is very little intracellular collagen. Images of untransfected FN-null-MEF cells are shown at right to confirm specificity of the GFP signal (bars = 10µm). **B)** Immunostaining using LF67 antibody for collagen α1(I) in FNKO-colGFP cells cultured without ascorbate and permeabilized with triton-X100. With permeabilization, the LF67 antibody penetrates into the cell and the intracellular collagen-GFP*tpz* co-localizes with immunostaining for α1(I) collagen (compare to figure 3C in the main manuscript, which shows staining without permeabilization). Insets show IgG stained control cultures (bar = 50µm).

Figure S6



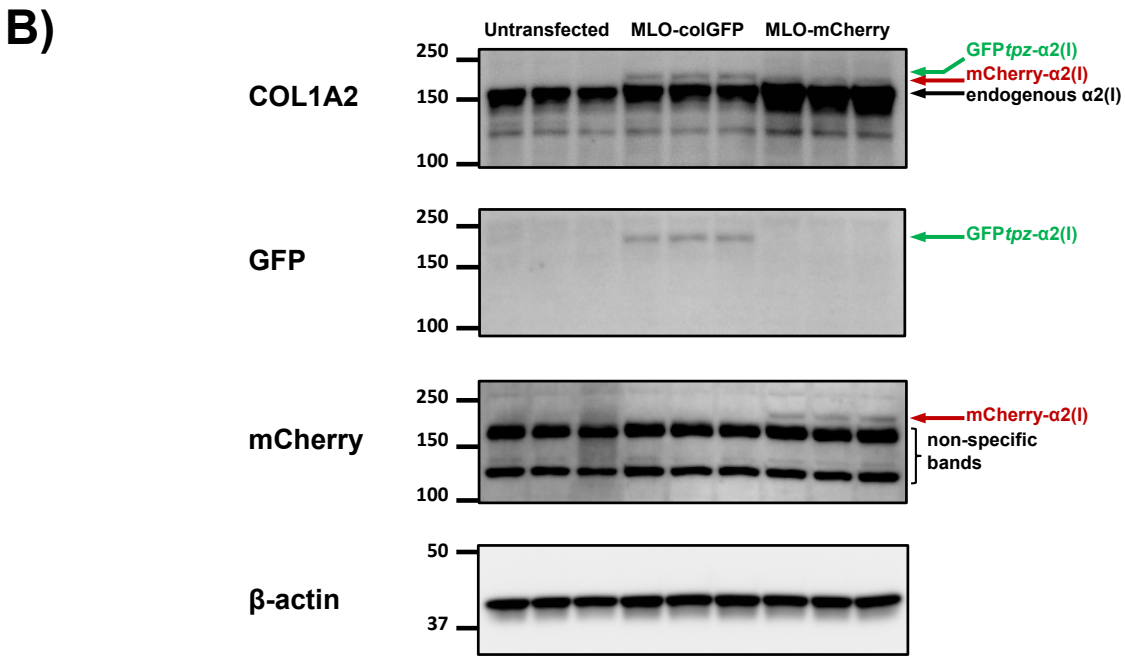


Fig. S6: In MLO-colGFP cells, the GFP*tpz*-Collagen is Intracellular Without Ascorbate and is Deposited Below the Cell Layer in the Presence of Ascorbate– A) Confocal vertical section and selected Z slices through MLO-colGFP cells cultured without ascorbate (upper panels) or with ascorbate (lower panels) and stained with the membrane dye DiD (red). Note that without ascorbate the GFP*tpz*-collagen signal (green) remains intracellular as shown in the vertical section and in all representative z-slices (z9, z19 & z24). In contrast, with ascorbate, the GFP*tpz*-collagen signal is localized in extracellular fibrils, which are predominantly deposited below the cell layer. The selected Z slices show that at the lower levels nearest to the growth surface (dashed line) only GFP*tpz*-collagen is present (z12). In the central level (z24) both cells and GFPtpz-collagen are present and in the upper level (z38) only cells are present. Images at left show untransfected parental MLO-A5 cells showing the specificity of the GFP signal. Bars = 10µm. **B)** Western blotting of whole cell lysates from untransfected parental MLO-A5 cells, MLO-colGFP and MLO-colCherry cells immunoblotted with antibodies against Col1a2, GFP, DsRed(mCherry) and β-actin. Note that the endogenous α2(I) procollagen runs at ~150kDa and that the GFP*tpz*-tagged and mCherry-tagged α2(I) procollagen bands run slightly higher and represent a minor fraction of the total α2(I) procollagen.

Figure S7

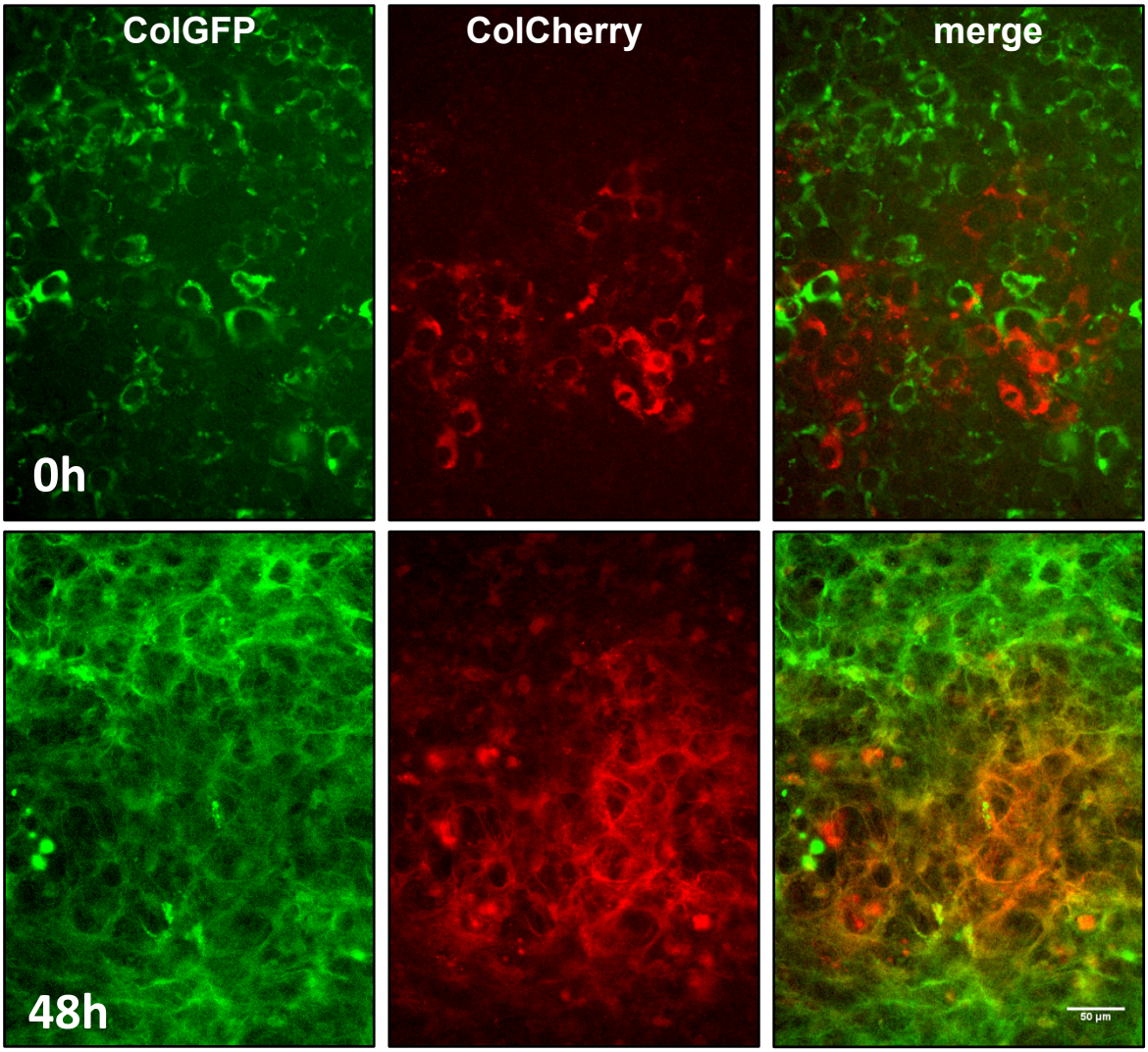


Fig. S7: Multiple Cells Contribute to Formation of Collagen Fibers- still frames from a representative time lapse movie of 1:1 co-cultures of MLO-colCherry and MLO-colGFP cell lines [see corresponding supplementary movie 3]. Ascorbate was added at the beginning of the movie (0h). Note that at 0h, the mCherry or GFP*tpz*-collagen are intracellular with a perinuclear localization. Individual mCherry or GFP*tpz*-collagen expressing cells can be distinguished. By 48h a well formed network of collagen fibrils has formed and in areas where both types of cells are present, the mCherry and GFP*tpz*-collagen is localized within the same fibrils. Bar = 50µm.

Figure S8

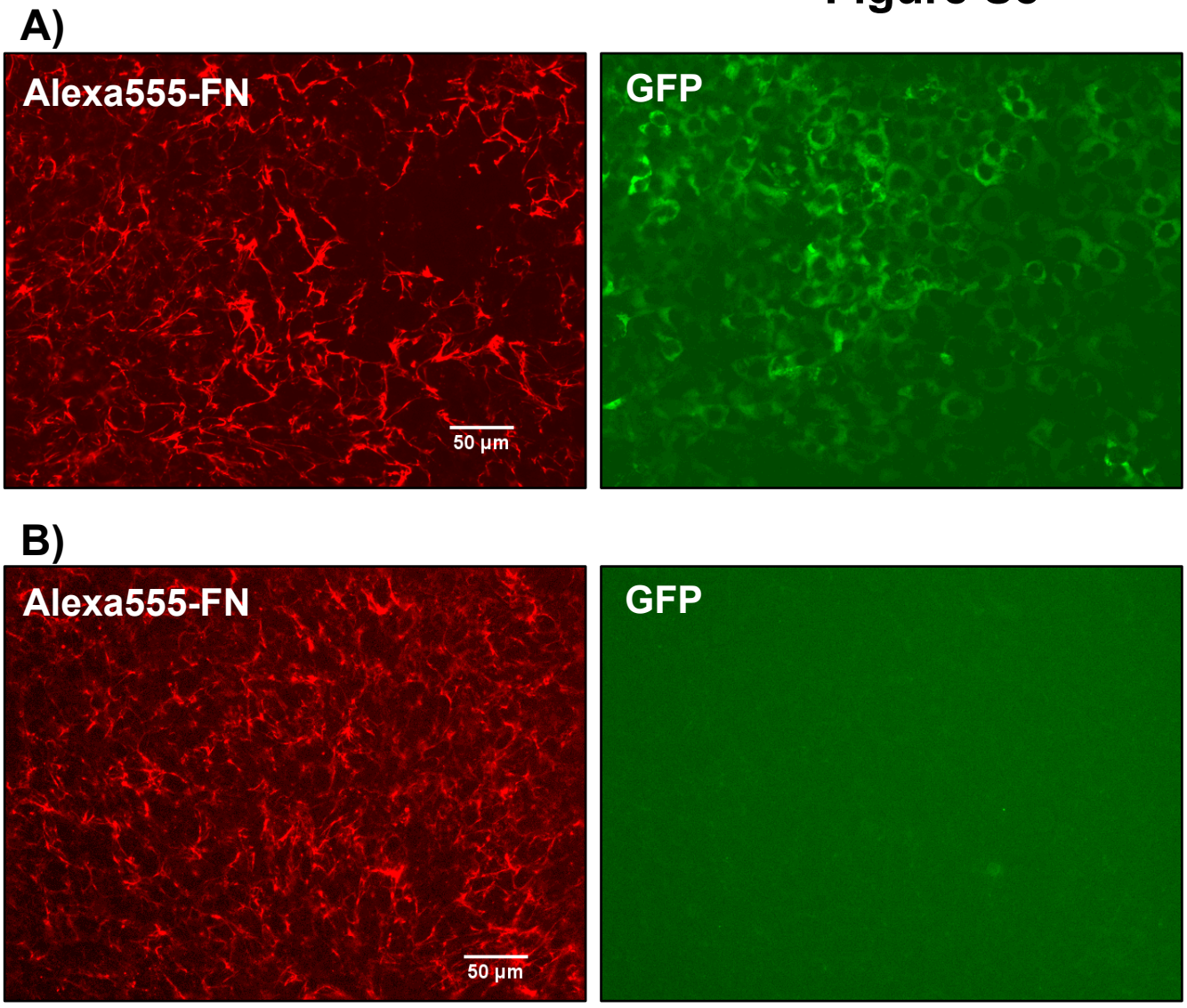


Fig. S8: Controls Showing no Bleedthrough of Alexa555-FN Signal into GFP Channel – A) FNKO-colGFP cells were cultured without ascorbate but with 2.5µg/ml Alexa55 labeled fibronectin and 7.5µg/ml unlabeled fibronectin. Note that a well formed fibronectin fibril network has assembled but collagen remains intracellular due to the lack of ascorbate. There is no bleedthrough of fibril-like image features from the Alexa555-fibronectin image into the green channel. **B)** Untransfected fibronectin null MEFs were cultured with 2.5µg/ml Alexa555-labeled fibronectin and 7.5µg/ml unlabeled fibronectin. Note that a well formed fibronectin network has assembled. There is negligible bleedthrough of fibril-like image features from the Alexa555-fibronectin image into the green (GFP) channel. Bar = 50µm.

LEGENDS FOR SUPPLEMENTARY MOVIES

Movie 1: Timelapse movie in MLO-colGFP cells showing that collagen fibers do not assemble in the absence of ascorbate. [LEFT PANEL: differential interference contrast (DIC) image of MLO-colGFP cells; CENTER PANEL: GFP*tpz* collagen; RIGHT PANEL: merged DIC/GFP image]. MLO-colGFP cells were cultured in the absence of ascorbate. Note the extensive cell motion and numerous cell mitoses. Also note that without ascorbate, the collagen remains intracellular throughout the 52h imaging period and is not assembled into fibrils. By 3h a cell labeled X divides into two daughter cells X_1 and X_2. The GFP*tpz*-collagen is shared equally between the daughter cells. Bar = 50μm.

Movie 2: Timelapse movie in MLO-colGFP cells showing collagen assembly with ascorbate supplementation. [LEFT PANEL: DIC image of MLO-colGFP cells; CENTER PANEL: GFP*tpz* collagen; RIGHT PANEL: merged DIC/GFP image]. MLO-colGFP cells were supplemented with ascorbate at the start of the movie. Note the extensive cell motion and numerous cell mitoses. Also note that with ascorbate, the collagen migrates towards the cell periphery in small vesicle-like structures by 3-8h (white arrowheads). A few sparse collagen fibers are visible by 10-14h and deposition increases throughout the movie until an extensive collagen network has been assembled. Note that the collagen fibers are continually stretched and contracted during their assembly due to the underlying cell motion. By 19h, a cell labeled Z divides into two daughter cells Z_1 and Z_2. By 19-20h cell Y divides into two daughter cells Y_1 and Y_2. In both cases, the GFP*tpz*-collagen is shared equally between the daughter cells. By 34h, a hole appears in the collagen network. A second hole appears by 35h and a third one appears by 39h (white arrows). These holes enlarge as the movie progresses. Bar = 50μm.

Movie 3: Time lapse movie in MLO-colGFP and MLO-colCherry co-cultures showing co-deposition of red and green collagen. [LEFT PANEL: GFP*tpz*-collagen; CENTER PANEL: mCherry-collagen; RIGHT PANEL: merged image of GFP*tpz*-collagen and mCherry collagen]. MLO-colGFP and MLO-colCherry cells were co-cultured and ascorbate was added at the start of the movie. Note the extensive cell motion throughout the movie. By 3-8h after ascorbate addition the collagen migrates into vesicle-like structures inside the cells. A few red or green collagen fibers are seen by 14h and a more extensive network by 24-58h. In locations where both red and green collagen producing cells are present, the collagen is deposited in the same fibrillar structures, which move together due to the underlying cell motion (see merged images in right panel). Bar = 50μm.

Movie 4 – Time lapse movie showing the dependence of collagen assembly on fibronectin. [LEFT PANEL: FNKO-colGFP cells cultured without fibronectin; RIGHT PANEL: FNKO-colGFP cells cultured with fibronectin]. FNKO-colGFP cells were cultured with ascorbate and with or without fibronectin supplementation. Both factors were added at the start of the movie. Note the extensive cell motion throughout the movie. By 3-8h after ascorbate addition the GFP*tpz*-collagen migrates into vesicle-like structures regardless of whether fibronectin is present. With fibronectin supplementation, collagen fibers begin to be deposited by 20h and accumulate through 46h. In contrast, without fibronectin supplementation only a few sparse collagen fibers are deposited. Bar = 50μm.

Movie 5 – Timelapse movie showing co-deposition of fibronectin and collagen. [LEFT PANEL: GFP*tpz*-collagen; CENTER PANEL: Alexa555-fibronectin; RIGHT PANEL: merged image of GFP*tpz*-collagen and Alexa555-FN]. FNKO-colGFP cells were cultured without fibronectin but with ascorbate prior to imaging. At the start of imaging the media was supplemented with fresh ascorbate and 2.5μg/ml Alexa555-fibronectin plus 7.5μg/ml unlabeled fibronectin for dual imaging of fibronectin and collagen assembly. Note that a few small fibronectin fibrils start to be assembled by 3h and continue to assemble throughout the 46h imaging period. GFP*tpz*-collagen fibers are co-deposited with fibronectin. Some of the GFP signal is not co-localized with fibronectin, which mainly represents intracellular collagen. Note that the collagen and fibronectin fibrils show identical motions during their assembly due to the underlying cell motion. Bar = 50μm.

Image processing for all movies was done in Image J. Image stacks for movies 1-3 were normalized and image stacks for movies 4 and 5 were background subtracted (50nm rolling ball). All image stacks were registered using the stackReg plugin, pseudocolored (if applicable), converted to RGB, compiled and annotated in Image J.