demonstrate the portion of phosphorylated paxillin over the total paxillin. Images without first antibodies (a and f) are shown as negative controls. For comparison, panels were captured with identical gain and iris value and processed in the same way.

Figure 4

Elevated Src family kinase activity in high-metastatic sublines.

A: Elevated Src family kinase activity in high-metastatic sublines. Src family kinases of low- and high-metastatic sublines and parental HuO9 cell lysates were immunoprecipitated by Src-2 antibody. Lysates of L12 and M112 immunoprecipitated with pre-immune rabbit serum were used as negative controls. Kinase activity was evaluated by phosphorylation of exogenous synthetic polypeptide poly[Glu-Tyr](4:1). The density of each smear (between 50 kDa and 150 kDa, area shown by a bracket) was quantified.

B: FAK or c-Abl of low- and high-metastatic sublines and parental HuO9 were immunoprecipitated by monoclonal antibodies. To evaluate kinase activity, exogenous synthetic polypeptide poly[Glu-Tyr](4:1) was used. The kinase activities were quantified according to the same method as described in Figure 4A.

C: Src family kinase inhibitor PP2 impairs the motility of high-metastatic sublines. Motility of low- and high-metastatic sublines and parental HuO9 cells was evaluated by migration assay as described in Materials and Methods. As for high-metastatic sublines, M112 and M132, motility in the presence of 10 μM of PP2 or 10 μM of PP3 was also evaluated. The cells at the lower side of the filters were stained by Giemsa's stain solution and visualized under microscope at a magnification of 200_. Each bar represents the mean number of cells ± SD counted in five fields.

D: Src family kinase inhibitor PP2 abolishes tyrosine phosphorylation of paxillin. High-metastatic subline M112 and M132 were treated with 10 mM of PP2 or PP3 for 30 min prior to cell lysis. Whole cell lysates were immunoblotted for anti-phospho-paxillin antibody, anti-paxillin antibody, and anti-α-tubulin antibody.

Figure 5

Cell migration was attenuated by knocking down of paxillin expression in high-metastatic sublines.

A: M112 subline was transfected with siRNA of paxillin or LacZ, or treated only with lipofection reagent ((-), as indicated bottom). Cells were lysed at 72 hr from the transfection and whole cell lysates were immunoblotted with the antibodies indicated. The density of each band is shown on the right.

B: M112 subline was transfected with siRNA or treated only with lipofection reagent as described above. Transfected cells were subjected to migration assay as described in Materials and Methods.

Figure 6

Overexpression of paxillin and elevation of Src family kinase activity synergistically enhance the motility of human osteosarcoma.

A: Cos-7 cells were transiently transfected with empty vector (VEC), GFP-paxillin (WT) or GFP-2F (Y31F, Y118F) mutant (2F). Cells were lysed at 48 hr from the transfection and immunoprecipitated with anti-GFP antibody. Immunoprecipitates were subjected to immunoblotting analysis by anti-phosphotyrosine antibody 4G10 and anti-paxillin antibody.

B: L12 cells were transiently transfected with empty vector (VEC), GFP-paxillin (WT) or GFP-2F mutant (2F). Whole cell lysates were immunoblotted for anti-phospho-paxillin antibody (Tyr 118) and paxillin antibody. GFP-paxillin and GFP-2F mutant were indicated (arrowhead a and

b).
C: L12 cells transfected with GFP-paxillin or GFP-2F mutant were chemically stained with phalloidin. GFP (a, b: green) and phalloidin (c, d: red) were visualized with confocal microscopy.
D: L12 cells transfected with empty vector, GFP-paxillin or GFP-2F mutant were subjected to migration assay as described in Materials and Methods.
E, F: L12 cells (L12 wt) or paxillin-FLAG expressing stable cells (L12 pax) were transiently transfected empty vector (VEC) or Fyn-FLAG (Fyn). Whole cell lysates were immunoblotted for the antibodies indicated. Fyn-FLAG (arrowhead a) and endogenous Fyn (arrowhead b) were shown in Figure 6E upper panel.
G: L12 cells or paxillin-FLAG expressing stable cells which were transiently transfected empty vector or Fyn-FLAG were subjected to migration assay as described in Materials and Methods.

Figure 1

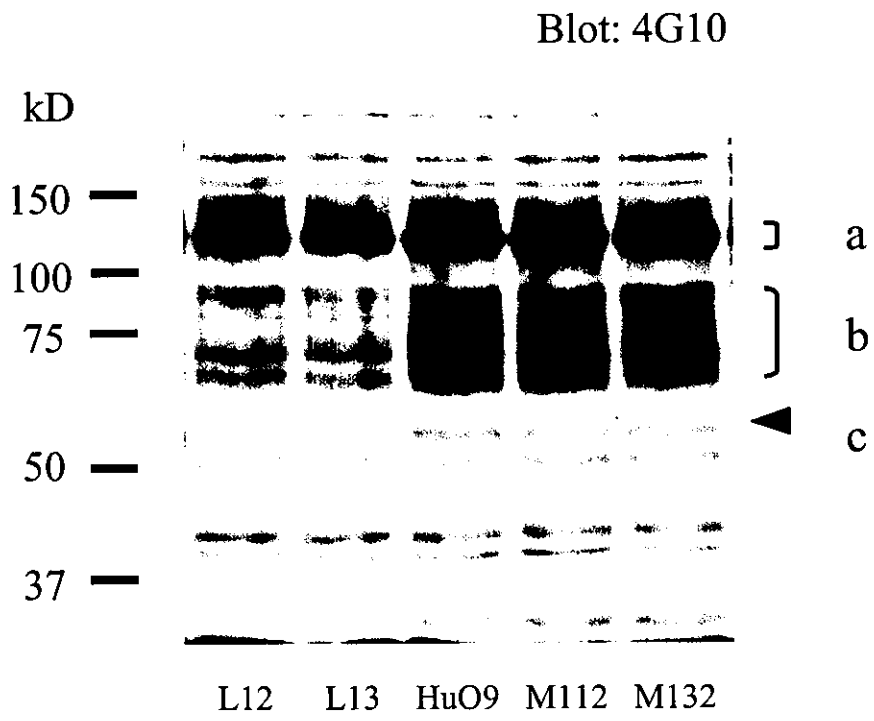
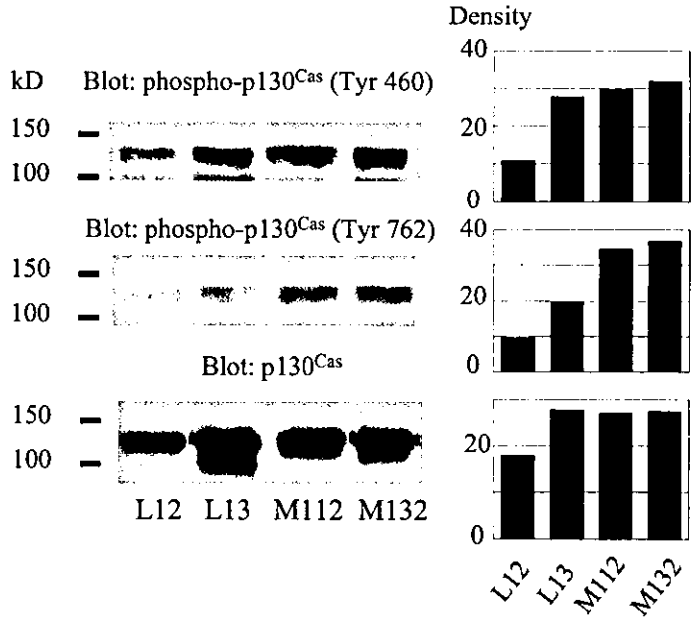
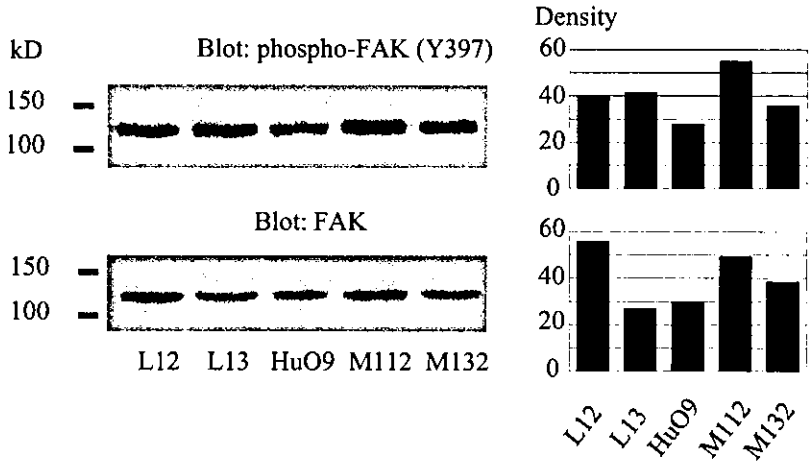


Figure 2

A


Density
kD
Blot: phospho-p130Cas (Tyr 460)
150
100
40
20
0
Blot: phospho-p130Cas (Tyr 762)
150
100
40
20
0
Blot: p130Cas
150
100
20
0
L12 L13 M112 M132
L12
L13
M112
M132

B


Density
kD
Blot: phospho-FAK (Y397)
150
100
60
40
20
0
Blot: FAK
150
100
60
40
20
0
L12 L13 HuO9 M112 M132
L12
L13
HuO9
M112
M132

C

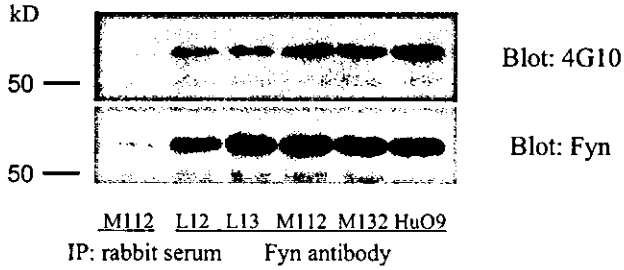

kD
50
50
Blot: 4G10
Blot: Fyn
M112 L12 L13 M112 M132 HuO9
IP: rabbit serum Fyn antibody

Figure 3

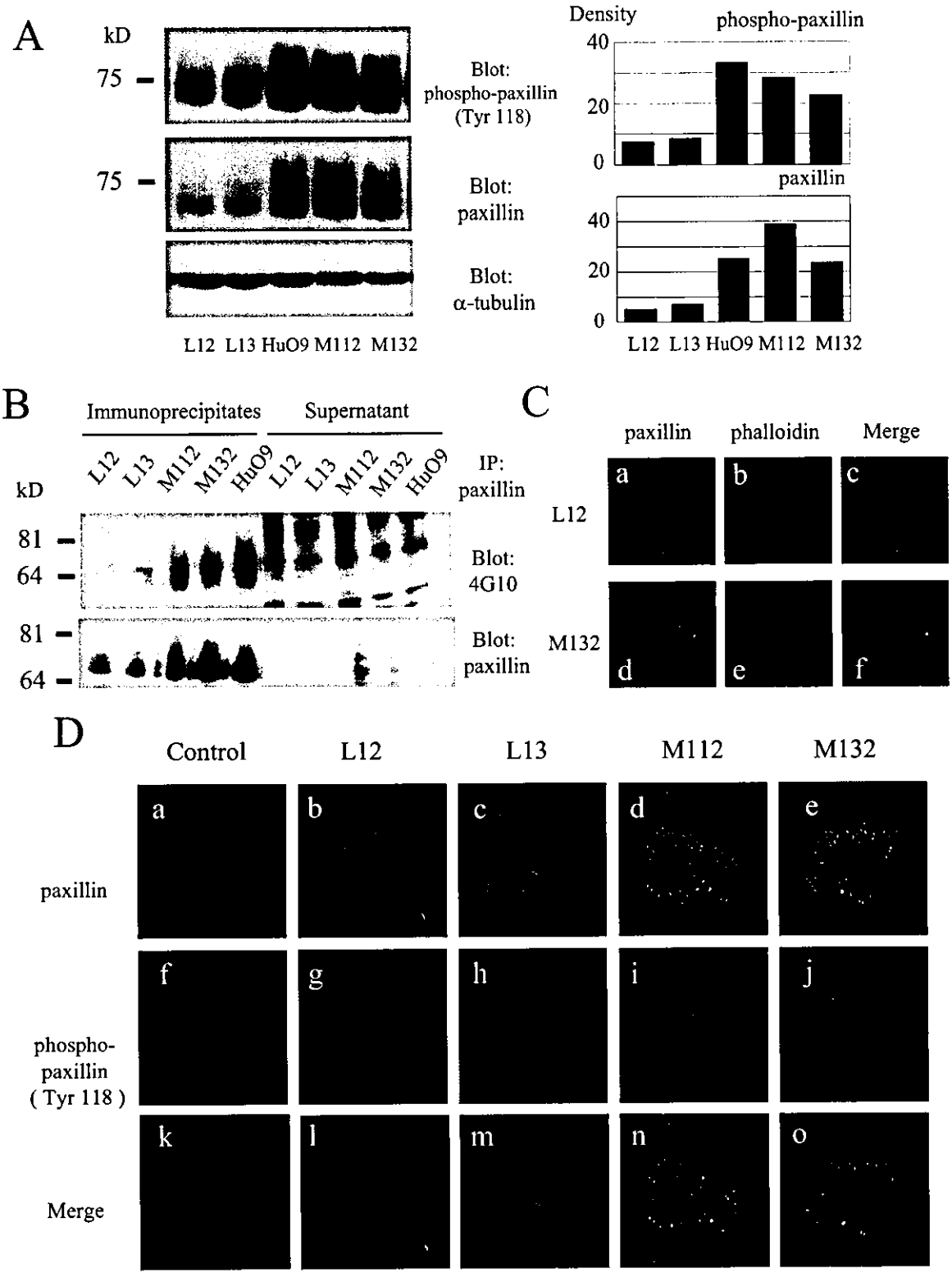

A
kD
75
75
Blot: phospho-paxillin (Tyr 118)
Blot: paxillin
Blot: α-tubulin
L12 L13 HuO9 M112 M132
Density
phospho-paxillin
40
20
0
paxillin
40
20
0
L12 L13 HuO9 M112 M132
B
Immunoprecipitates
Supernatant
L12 L13 M112 M132 HuO9 L12 L13 M112 M132 HuO9
kD
81
64
81
64
IP: paxillin
Blot: 4G10
Blot: paxillin
C
paxillin
phalloidin
Merge
L12
M132
a
b
c
d
e
f
D
Control
L12
L13
M112
M132
paxillin
phospho-paxillin (Tyr 118)
Merge
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o

Figure 4

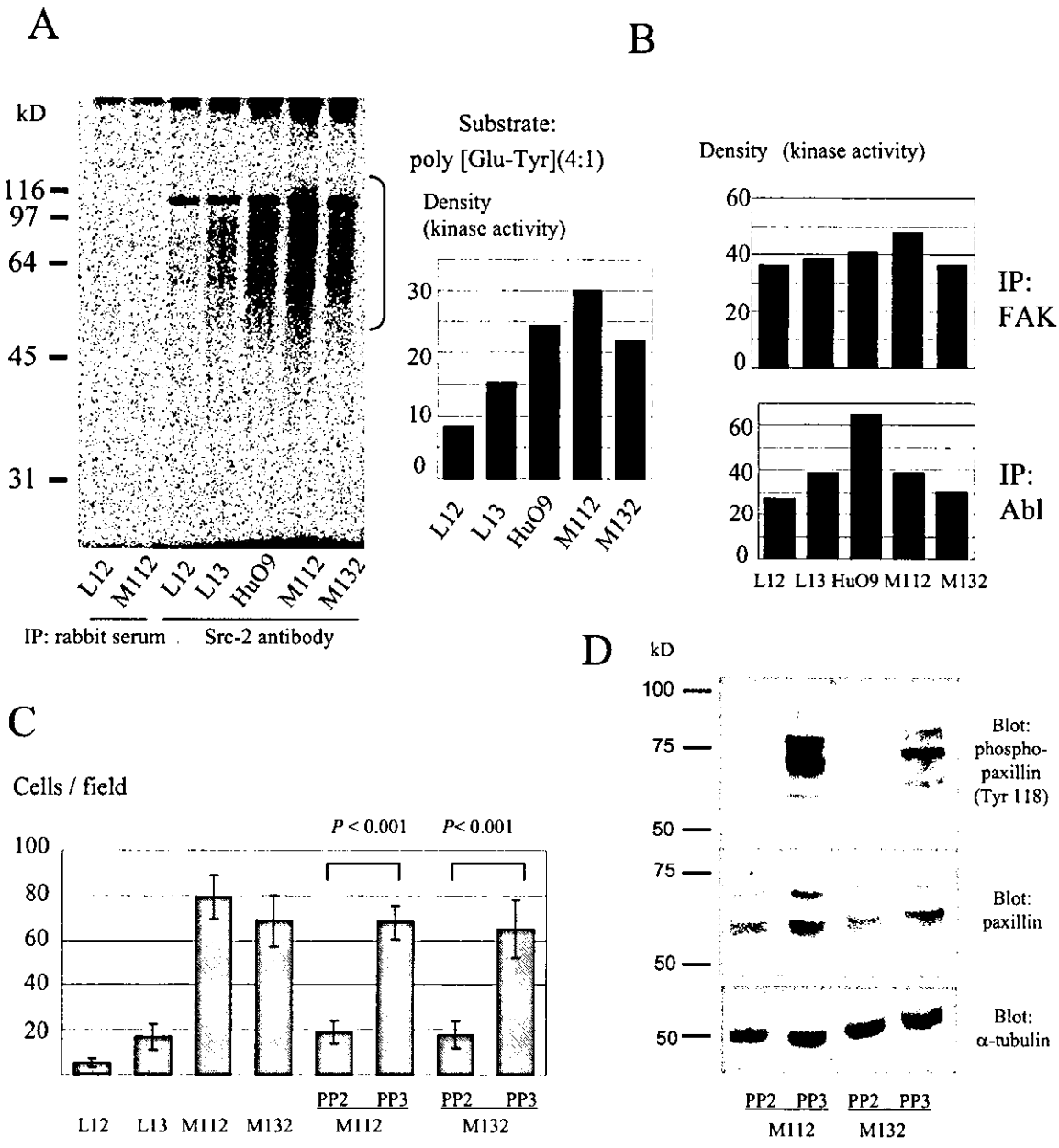
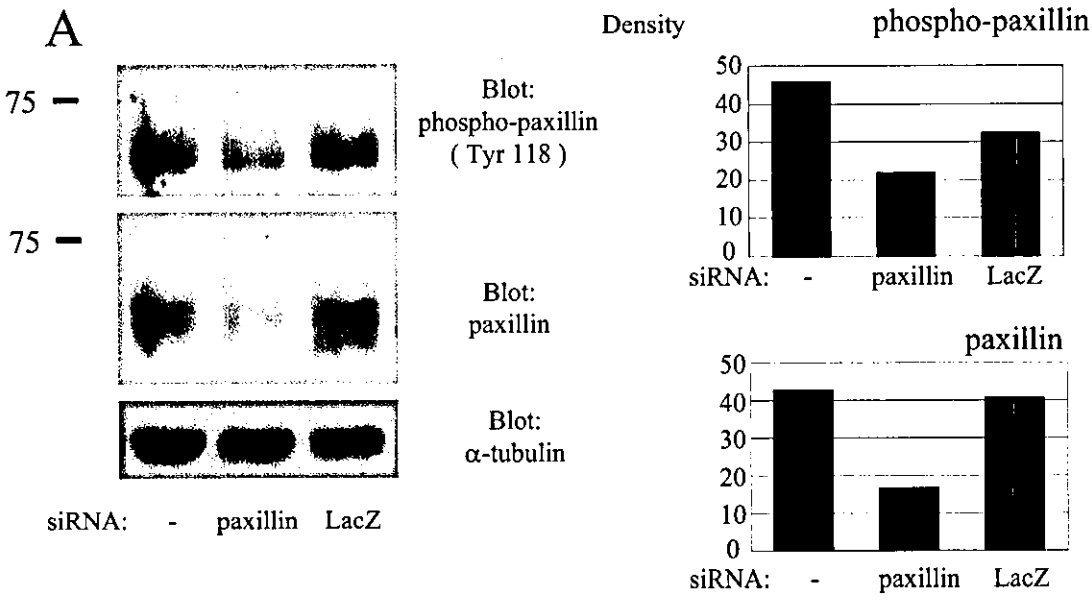

A
kD
116
97
64
45
31
L12
M112
L12
L13
HuO9
M112
M132
IP: rabbit serum
Src-2 antibody
Substrate:
poly [Glu-Tyr](4:1)
Density
(kinase activity)
30
20
10
0
L12
L13
HuO9
M112
M132
B
Density (kinase activity)
60
40
20
0
IP:
FAK
IP:
Abl
L12 L13 HuO9 M112 M132
C
Cells / field
P < 0.001
P< 0.001
100
80
60
40
20
PP2
PP3
PP2
PP3
L12
L13
M112
M132
M112
M132
D
kD
100
75
50
75
50
50
Blot:
phospho-
paxillin
(Tyr 118)
Blot:
paxillin
Blot:
α-tubulin
PP2
PP3
PP2
PP3
M112
M132

Figure 5


A
75
75
Blot:
phospho-paxillin
(Tyr 118)
Blot:
paxillin
Blot:
α-tubulin
siRNA: - paxillin LacZ
Density
phospho-paxillin
50
40
30
20
10
0
siRNA: - paxillin LacZ
paxillin
50
40
30
20
10
0
siRNA: - paxillin LacZ

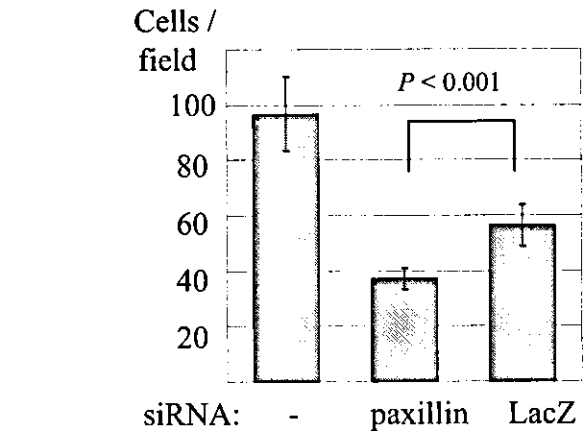
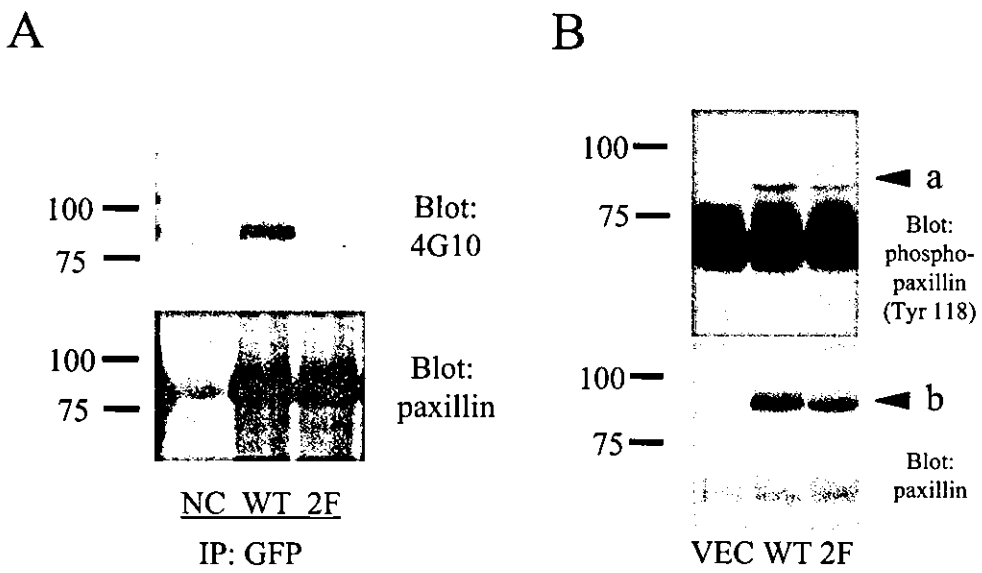

B
Cells /
field
P < 0.001
100
80
60
40
20
siRNA: - paxillin LacZ

Figure 6


A
100
75
Blot:
4G10
100
75
Blot:
paxillin
NC WT 2F
IP: GFP
B
100
75
a
Blot:
phospho-
paxillin
(Tyr 118)
100
75
b
Blot:
paxillin
VEC WT 2F

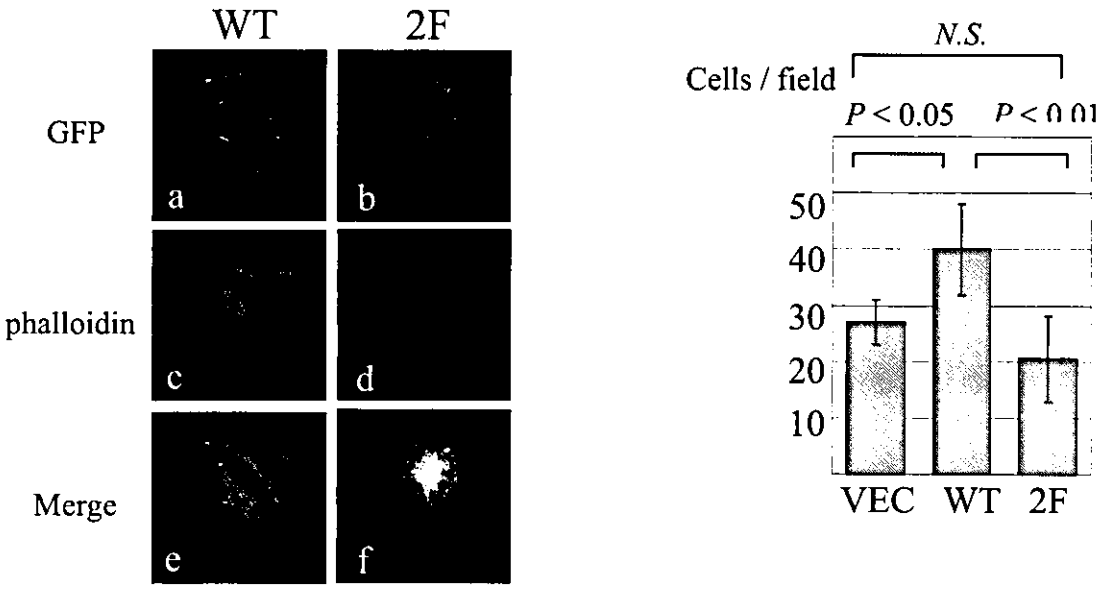
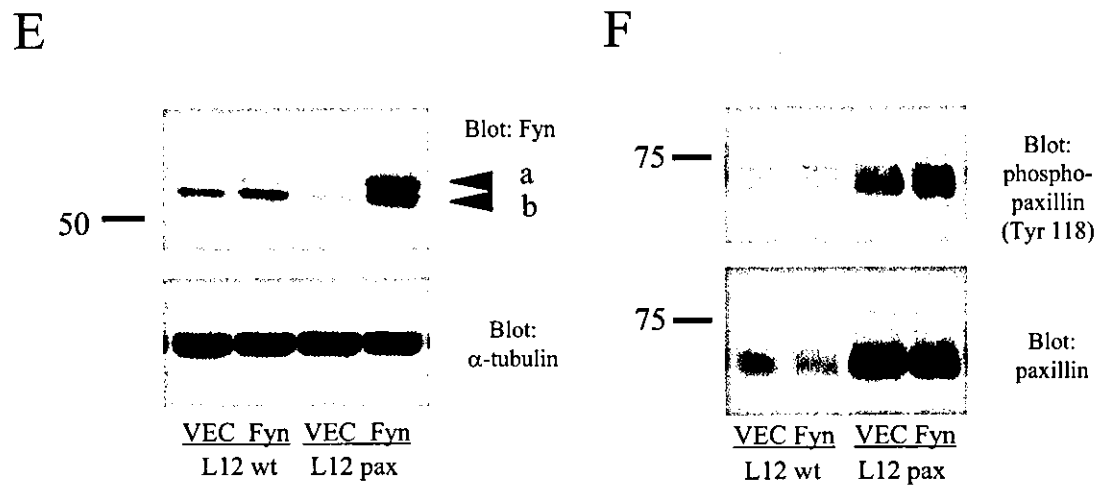
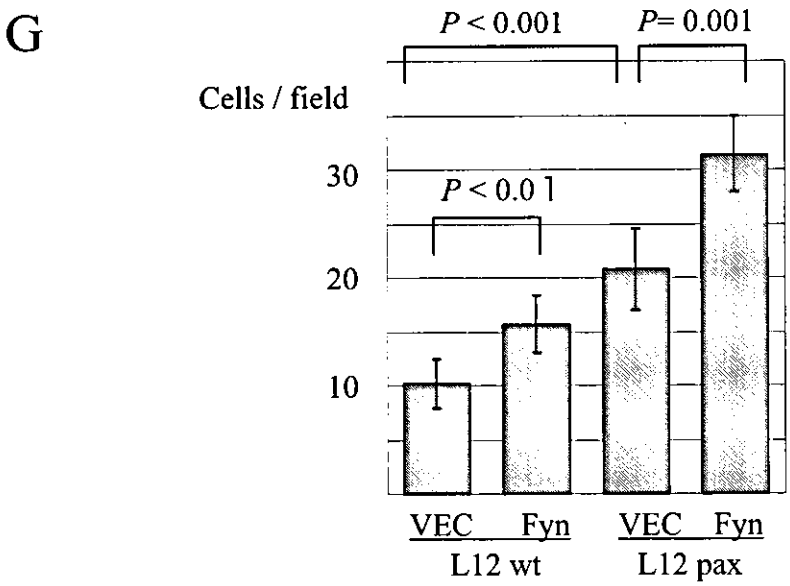
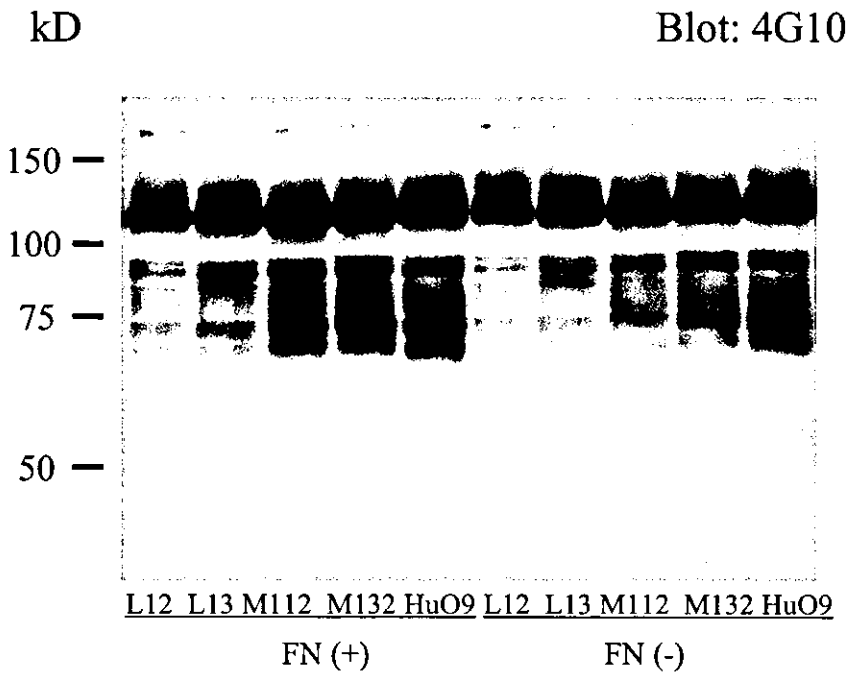

C
WT
2F
GFP
phalloidin
Merge
a
b
c
d
e
f
D
N.S.
Cells / field
P < 0.05
P < 0.01
50
40
30
20
10
VEC
WT
2F

Figure 6


E
F
Blot: Fyn
a
b
50
Blot:
α-tubulin
VEC Fyn VEC Fyn
L12 wt L12 pax
75
75
Blot:
phospho-
paxillin
(Tyr 118)
Blot:
paxillin
VEC Fyn VEC Fyn
L12 wt L12 pax


G
P < 0.001
P= 0.001
Cells / field
30
20
10
P < 0.01
VEC Fyn VEC Fyn
L12 wt L12 pax

Supplemental Figure 1



Supplemental Figure 2